Promising miRNA Biomarkers for Predicting the Response to the Interferon Combined Therapy in HCV-4 Egyptian Patients

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ABSTRACT

HCV infection is the major factor for chronic hepatitis. Interferons (IFNs) are large family of multifunctional secreted proteins which are used for HCV treatment. Large numbers of Egyptian patients are not responding to the combined interferon/ribavirin therapy. MicroRNAs (miRNAs) are class of post transcription regulators that play important role in HCV infection. The current study aimed to find a predictive measure for response to interferon therapy using miR-20a, miR-155, miR-21 and miR-196b with evaluating MxA gene expression as a positive control for interferon action. One hundred patients, 50 responders and 50 non-responders with chronic HCV and 20 healthy volunteers were included in this study. MiRNAs and MxA-mRNA were isolated from serum and blood sequentially and their expression levels were quantified using RT-qPCR. All the 4 miRNAs; miR-20a, miR-21, miR-155, and miR-196b were up regulated in the serum of the non-responders compared to responders and controls. These data were confirmed by the inhibition of MxA-mRNA levels in the blood of the non-responders compared to controls and responders. In conclusion, the circulating miRNAs; miR-20a, miR-21, miR-155 and miR-196b in serum can be promising biomarkers for predicting the response of HCV-4 Egyptian patients to IFN therapy during the combined IFN treatment.

Key words: *MicroRNA, MxA-mRNA, RT-qPCR.*

INTRODUCTION

Hepatitis C virus (HCV) is a positive strand RNA virus from flaviviridae family with 170-200 million peoples worldwide are estimated to be infected, and it is the major cause of chronic hepatitis (Shrivastava *et al.*, 2015). HCV genotype 4 is a very heterogeneous genotype that accounts for more than 90% of the HCV infection in Egypt (Abdel-Ghaffar *et al.*, 2015). Interferon alpha (IFN-α) treatment was the main method for HCV-4 treatment for the last 20 years. Pegylated IFN-α (PEG-IFN-α) was developed to ensure sustained exposure with once weekly dose which offers improved convenience (Farrell, 2007). Nearly 42-50% of the patients are non-responders to PEG-IFN-α treatment or relapse after the therapy is discontinued making this the main challenge in HCV-4 patients treatment (Shepherda *et al.*, 2015).

HCV therapy is currently undergoing a revolution, with several new treatments have been approved by the United States Food and Drug Administration (FDA), and many other treatments are in phase II or III clinical trials,

including direct antiviral agents (DAAs). Therapy for HCV-4 patients depends on triple combined therapy of PEG-IFN, sofosbuvir and ribavirin with an excellent sustained virological response (SVR) rate of 96% (Al-Judaibi, 2015).

MicroRNAs (miRNAs) are a class of post transcription regulators, expressed from non-coding gene and consist of ~ 22 nucleotides (Felekkis *et al*., 2010). They silence the protein coding transcript, and are involved in nearly all developmental and pathological processes in animals. Their biogenesis is under tight control and their dysregulation cause many human diseases (Minju and Narry, 2014). MicroRNAs can exert a profound effect on HCV replication. HCV infection can trigger changes in the cellular miRNA profile, which may ultimately contribute to the outcome of viral infection (Lindow and Kauppinen, 2012). Conaco *et al*, (2006) indicated that miRNAs may be involved in IFN-responsiveness to viral infection.

The search for noninvasive biomarkers for diagnosis of diseases has become a rapidly growing area of clinical research (TenCate *et al*., 2010). The aim of the present study was to examine if some miRNAs or even one specific miRNA might be sufficient to differentiate the responders for the combined pegylated interferon alpha /ribavirin therapy from non-responders patients.

MATERIALS AND METHODS

Study design

One hundred patients (50 responders and 50 non-responders) with ages ranging from 20-60 years with HCV after 12 weeks of the combined pegylated interferon alpha /ribavirin therapy and twenty healthy controls were included in this study. HCV patients were treated by subcutaneous injection with pegylated interferon alpha-2a (PEGASYS; Hoffmann-La Roche, Basel, Switzerland) 180 µg/kg once/week plus ribavirin 1000-1200 mg/kg (for body weight <75kg or ≥75kg, respectively). According to the HCV viral load, after 12 weeks of treatment, patients were divided into responders and non-responders. The inclusion criteria was: patients with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels ranging from 20-82 IU/l. The exclusion criteria was: HCV infected patients under the age of 20 and over the age of 60 years, co-infected with human immunodeficiency virus (HIV), autoimmune hepatitis or hemochromatosis were excluded from the prospective study. Written informed consent obtained from all subjects in this study was approved by the Ethics Committee of Theodor Bilharz Research Institute (TBRI)) according to the institutional committee for the protection of human subjects and adopted by the 18th world medical assembly, Helsinki, Finland.

Samples handling and preparation

From each patient and healthy control, 2x 5ml venous blood were drawn into two tubes, one containing ethylenediamine-tetraacetic acid (EDTA) which was aliquoted and stored at -20°C and the other sample was drawn in non-additive tube and left to clot at room temperature for 30 min, centrifuged at 2000 rpm at 4°C for 10 min and then serum was removed, aliquoted and stored at -80°C (Tuck *et al*., 2009).

Biochemical Investigations

Laboratory tests including the liver associated enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) which are sensitive indicators in recognizing hepatocellular diseases were performed to the HCV infected patients treated with combined

pegylated interferon alpha /ribavirin and healthy controls. All analyses were performed in duplicates.

HCV viral load quantification

HCV RNA extraction was performed using Abbott *m*sample preparation system kit (Abbott Molecular, Inc., Des Plaines, Illinois, USA) and the viral loads were measured by real-time reverse transcription polymerase chain reaction using 7500 RT-PCR system (AB Applied Biosystems, Foster City, California, USA). The HCV-RNA in serum was amplified using the Abbott Real-time HCV Amplification Reagent Pack (Abbott Molecular, Inc., Des Plaines, Illinois, USA) according to the manufacturer's instructions.

MiRNAs extraction and RT reaction

Total RNA with preserved miRNAs was extracted from 200 µl serum with mirVana™ PARIS™Kit (Ambion, Austin, TX, USA). Reverse transcription was performed with 5µl of total RNA in a final volume of 15 µl using the TaqMan® MicroRNA Reverse Transcription Kit (AB Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

MxA cDNA synthesis

MxA-mRNA was extracted from 100µl blood using magnetic bead extraction method (Abbott Molecular, Inc., Des Plaines, IL, USA). The MxA-cDNA was synthesized from total RNA using the High Capacity Reverse Transcription Kit (AB Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Quantitative analysis

The expression pattern of mir-20a, mir-155, mir21, mir-196b and MxA-mRNA were evaluated using qRT-PCR analysis by StepOne™ Real-Time PCR in which ready-made TaqMan MicroRNA assay and ready-made MxA assay (AB Applied Biosystems, Foster City, CA, USA) were used according to the manufacturer's protocol. All miRNAs and MxA-mRNA expression values were evaluated using relative quantification analysis by ΔΔCt method (Akin *et al*., 2012) and normalized to *C.elegans* mir-39 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) respectively.

Statistical analysis

Data analysis was carried out using Prism 5 software, version 5.00 (GraphPad Software, San Diego, CA, USA). Data were presented as the mean ± standard error (± SE). MiRNAs expression in serum and MxA-mRNA expression in blood were calculated using the formula ($RQ=2^{\wedge -\Delta\Delta CT}$). Comparisons of quantitative variables were performed between two groups by using Mann Whitney U-test (GraphPad Software, San Diego, CA, USA), and multiple comparisons between more than two groups have been conducted by Kruskal Wallis ANOVA test (GraphPad Software, San Diego, CA, USA). The Spearman rank order correlation test was used to examine correlation relationships. The P value <0.05 was considered statistically significant, P value ≤ 0.01 was considered highly significant and P value ≤ 0.0001 was considered very highly significant. Sensitivity and specificity of each test were performed using the receiver operating characteristic (ROC) curve to determine the optimum cut off value for the studied diagnostic markers. Accuracy was measured by the area under the ROC curve (AUC). An area of 1 represented a perfect test and an area of 0.5 represented a worthless test.

RESULTS

Laboratory characterization of HCV patients and control group

Data presented in Table (1) indicate the analysis performed among the 100 HCV patients (responders and non-responders) in terms of ALT, AST, age and viral load. A very highly significant difference was observed between the control group, responders and non-responders in terms of liver function activities, ALT ($p<0.0001$) and AST ($p<0.0001$) and no significant difference in terms of age and viral load (at week zero before the treatment).

Expression pattern of miR-20a, miR-155, miR-21 and miR-196b in responders, non-responders and healthy controls

Fig. (1) shows the expression analysis of miR-20a, miR-155, miR-21 and miR-196b between responders and non-responders compared to controls. Non-responders had the highest expression values compared to responders and controls. MiR-155 and miR-21 showed very highly significant differences with P=0.0001 and P=0.0007 sequentially, miR-196b showed a highly significant difference (P=0.006) and miR-20a showed positive significant difference (P=0.023) among responders, non-responders and controls.

Correlation analysis between miR-20a, miR-155, miR21 and miR-196b

As shown in Fig. (2) very strong positive correlations between miR-20a and miR-21($P<0.0001$) and between miR-196b and miR-155($P\leq0.001$), strong positive correlations between miR-20a and miR-155(P=0.005) and miR-21 and miR-155(P=0.004), positive significant correlation between miR-196b and miR-20a (P=0.02) and non-significant correlation between miR-196b and miR-21 in HCV patients (responders and non-responders).

Expression pattern of MxA-mRNA in responders, non-responders and healthy controls

MxA-mRNA expression was quantified in blood of all subjects using qRT-PCR. There were a significant differences in MxA-mRNA expression levels among responders with P= 0.001, non-responders with p= 0.002 and controls with P= 0.001.

Table (1): Characteristics of HCV patients (responders and non-responders) and controls.

	Controls n= 20	Responders n=50	Non-responders n=50	P value
Age, years	39.45±2.38	42.15±1.03	38.26±1.07	NS
Males	12 (60%)	27 (54%)	18 (36%)	NS
Females	8(40%)	23 (46%)	32 (64%)	NS
ALT (IU/l)	22.30±1.00	36.96±1.30	45.48±1.16	<0.0001***
AST (IU/l)	27.86±0.70	33±0.87	44.83±1.12	<0.0001***
Log HCV PCR	------	2.364±0.04	2.38±0.004	NS

Values are represented as mean ± S.E, (n); number of patients, NS; non-significant,
**** very highly significant at P≤ 0.001.*

Table (2): Correlation analysis between microRNAs and MxA-mRNA expressions in responders and non-responders patients.

	MxA and responders	
	Spearman's rank (*rs*)	**P value**
miR-20a	-0.40	0.035*
miR-155	-0.31	NS
miR-21	-0.36	NS
miR-196b	-0.55	<0.0001***
	MxA and non-responders	
	Spearman's rank (*rs*)	**P value**
miR-20a	-0.61	<0.0001***
miR-155	-0.54	0.0002***
miR-21	-0.62	<0.0001***
miR-196b	-0.45	0.0049**

*NS; non-significant., *Significant at P ≤0.05, **highly significant at P≤0.01, ***very highly significant at P≤0.001.*

Table (3): The different parameters for the validation of miRNAs and MxA-mRNA diagnostic test.

Parameters	**Mir-20a**	**Mir-155**	**Mir-21**	**Mir-196b**	**MxA-mRNA**
Sensitivity	70%	83.44%	85 %	73.68%	75%
Specificity	60%	74%	72.41%	65.38%	83.33%
Cut off value	>1.0	>2.26	>1.57	>2.49	>8.25
PPV	63.64%	74.15%	86.36%	63.52%	76.51%
NPV	66.67%	83.44%	70.15%	75.22%	82.15%
AUC	0.667	0.831	0.805	0.756	0.789
P value	0.02*	<0.0001***	0.0003***	0.003**	0.0002***

*Positive predictive value (PPV), Negative predictive value (NPV), Area under curve (AUC), *significant at P ≤0.05, **highly significant at P≤0.01, ***very highly significant at P≤0.001.*

Correlation analysis between miRNAs and MxA-mRNA expressions

As shown in Table (2) responder patients had very strong inverse correlation between MxA-mRNA and miR-196b (P<0.0001), inverse significant correlation between MxA-mRNA and miR-20a (P=0.035) and non-significant correlation between MxA-mRNA and miR-155 and miR-21. However, in non-responder patients data revealed very strong inverse correlations between MxA-mRNA and miR-20a (P<0.0001), miR-21(P<0.0001) and miR-155 (P=0.0002) and a strong inverse correlation between MxA-mRNA and miR-196b (P=0.0049).

Validation of miRNAs and MxA-mRNA as Diagnostic biomarkers

The receiver operating characteristic (ROC) curve analyses display the diagnostic power of the 4 miRNAs and MxA-mRNA in predicting the response to combined pegylated

interferon alpha /ribavirin therapy in HCV patients when analyzed as a single marker. Data in Table (3) show the different parameters analyzed for miR-20a, miR-155, miR-21, miR-196b and MxA-mRNA between responders and non-responders. A significant difference in the expression of miR-20a, miR-155, miR-21, miR-196b and MxA-mRNA with P value 0.02, <0.0001, 0.0003, 0.003 and 0.0002, respectively was observed.

DISCUSSION

This study aimed to evaluate miRNAs as diagnostic biomarkers for predicting response to the combined pegylated interferon alpha /ribavirin therapy. The clinical data among 100 patients were analyzed and there were no significant difference in terms of age and viral load between HCV patients and healthy controls, but there was strong positive correlation between AST and ALT levels in HCV patients compared to healthy controls which indicate that the liver is affected.

The present study showed a very highly significant increase in the expression of miR-21 in non-responders compared to responders and controls. The up-regulation in the expression levels of miR-21 in HCV-4 patients could be due to the increased proliferation of liver cells during HCV infection and/or during the late stages of fibrosis (Marquez *et al.*, 2010). MiR-21 is induced within several h after IFN exposure and induction is clearly dose dependent, in *in-vitro* multiple cell types (Chuan *et al.*, 2010). Thus miR-21 is an IFN target gene, and our results confirm these data in an *in-vivo* study on human serum.

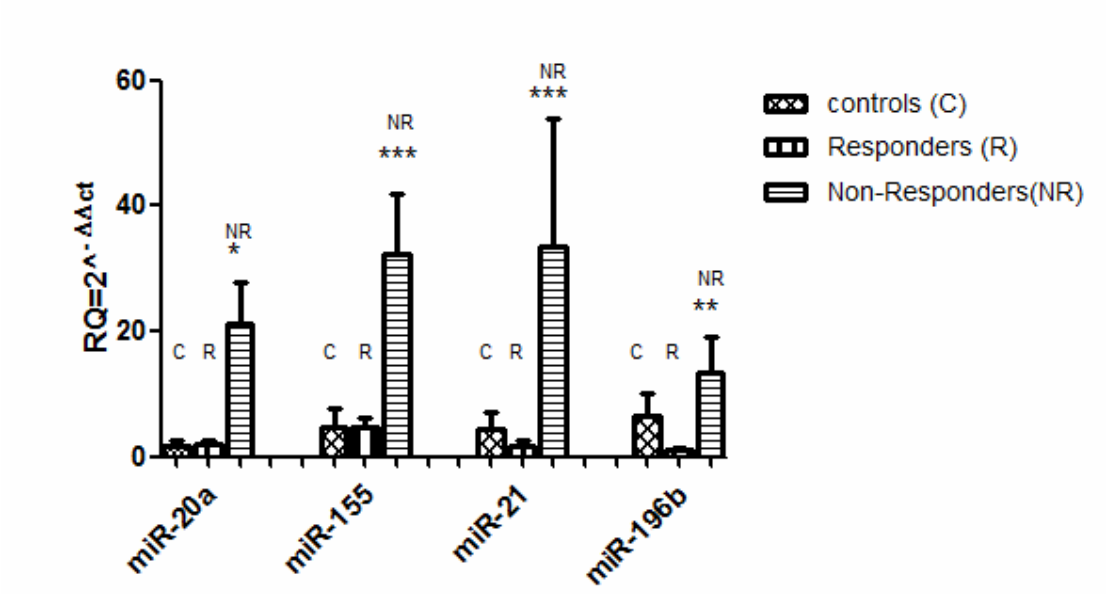

Fig. (1): Histogram of real-time qPCR of miR-20a, miR-155, miR-21 and miR-196b expression levels. Each column represents the relative amount of miRNAs. Data presented as relative quantification (RQ) based on 2^ $^{(-\Delta\Delta Ct)}$ method and representative of four independent experiments, C; controls, R; responders and NR; non-responders. *Significant at p ≤0.05, **highly significant at p≤0.01, ***very highly significant at p≤0.001.

MiR-155 is a common target of a broad range of inflammatory mediators. It promotes T-cell mediated tissue inflammation by regulating the interferon signaling (Baumjohann and Mark-Ansel, 2013). HCV infection induces miR-155 expression which induces hepatocytes proliferation and tumorigenesis (Zhang *et al*., 2012). According to the HCV genotype, miR-155 has shown an increase in the serum, PBMCs and liver tissues of HCV genotypes 1, 2 and 3 CHC infected patients (Bala *et al*., 2012). Our *in-vivo* study has demonstrated the negative correlation between miR-155 and the response to interferon therapy in HCV-4 patients. MiR-155 was very highly significantly increased in the serum of non-responder patients compared to responders and healthy controls. These data suggest that miR-155 inhibits the response of HCV patients to the combined pegylated interferon alpha /ribavirin therapy.

MiR-20a is known for its oncogenic role in several carcinogenesis (Mogilyansky and Rigoutsos, 2013). The correlation between miR-20a and HCC is well known, miR-20a is decreased in HCCs and correlates with HCC recurrence and prognosis (Fan *et al*., 2013). However, the role of miR-20a in responding to interferon therapy is not well understood. To our knowledge, the present study is the first in finding that the expression level of miR-20a was significantly increased in non- responders compared to responders and healthy controls. This may suggest that miR-20a role in HCV infection is negatively affecting the combined interferon therapy.

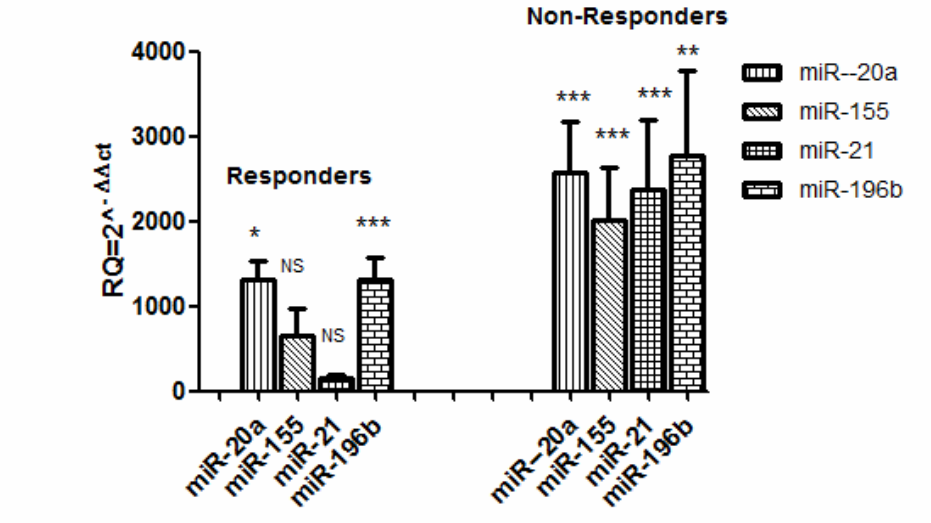

Fig. (2): The correlation analysis among miR-20a, miR-155, miR-21 and miR-196b expressions in HCV patients (responders and non-responders). Data are presented as mean of relative quantitation (RQ = $2^{\wedge\ (-\Delta\Delta CT)}$). NS; non-significant, *Significant at p ≤0.05, **highly significant at p≤0.01, ***very highly significant at p≤0.001.

MiR-196b contains putative recognition sites within HCV genome (Estrabaud *et al*., 2015). The direct effect of miR-196b on virus replication has been suggested on the basis of its perfect complementarity with the nonstructural 5A (NS5A) protein coding region of the viral genome (Pedersen *et al*., 2007). The present study found that miR-196b was highly significantly expressed in the serum of non-responders compared to responders and healthy controls. This may suggest that the alteration in miR-196b expression due to HCV infection may inhibit signaling and/or production of interferon and results in non-response to the combined IFN therapy.

A correlation analysis between miRNAs was performed. Significant positive correlations were observed between miR-20a and miR-21, miR-20a and miR-155, miR-21 and miR-155, miR-196b and miR-155 and miR-196b and miR-20a, while there was no correlation between miR-196b and miR-21. The correlation results indicated that there might be common regulators for these miRNAs expression. A further study between miRNA and mRNA expression profiles may give an opportunity to study the effects of gene expression between miRNAs and their target genes.

MxA, the well characterized IFN type I inducing gene was measured as positive control for IFN action (Scagnolari *et al*., 2010). MxA-mRNA expression levels were decreased in serum of non-responders compared to responders and healthy controls, which indicates a functional inhibition in the IFN pathway.

In the current study, the correlations between MxA-mRNA expression and miRNAs expression levels in the responders patients showed significant inverse correlation between MxA-mRNA and miR-20a and miR-196b and non-significant correlation with miR-155 and miR-21. However, non-responders patients showed significant inverse correlations among MxA-mRNA and miR-20a, miR-155, miR-21and miR-196b.

The diagnostic performance of miR-20a, miR-155, miR-21, miR-196b from responders and non-responders was evaluated by receiver operating characteristic (ROC) curve analysis. The ROC curve of miR-155, miR-21 and miR-196b reflected a strong ability to distinguish between non-responders and responders with an AUC of 0.83, 0.80 and 0.75 respectively, while miR-20a showed a moderate ability to distinguish between non-responders and responders with an AUC of 0.67. In addition, the diagnostic performance of MxA-mRNA reflected a strong separation between non-responders and responders with an AUC of 0.78.

In conclusion, all the studied miRNAs; miR-20a, miR-155, miR-21 and miR-196b were differentially expressed in serum of non-responders patients to the combined pegylated interferon alpha /ribavirin therapy and could differentiate non-responders from responders patients with cut off value for responding to the combined pegylated interferon alpha /ribavirin therapy >1, >2.26, >1.57 and >2.49, respectively. MxA-mRNA expression levels support these data by differentially being expressed in the blood of non-responders patients. This study recommended evaluating the expression levels of miR-155 with MxA-mRNA as a diagnostic biomarker to predict the responding of HCV-4 Egyptian patients to the pegylated interferon alpha /ribavirin therapy with cut off value > 2.26 and > 8.25 respectively. A deeper analysis of these miRNAs activity, target and interaction with the different component of the interferon pathway may help to elucidate the mechanisms of non-responders to interferon based

combined therapy. They can also be applied for developing novel anti-viral therapy for HCV-4 patients.

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الملخص العربي

الميكرو – رنا كمؤشرات حيوية واعدة للتنبؤ بإستجابة المرضى المصريين المصابين بفيروس الالتهاب الكبدى الوبائى سى – 4 للعلاج بالانترفيون المدمج

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يعد فيروس إلتهاب الكبد الوبائى سي هو العامل الرئيسى لإلتهاب الكبد المزمن و يعد الانترفيرون واحد من البروتينات المسئولة عن الدفاع ضد الفيروسات و يستخدم لعلاج فيروس سى. يوجد في مصر أعداد كبيرة من المرضى غير المستجيبين للعلاج بمزيج الانترفيرون ألفا المبيجل و الريبافرين. الميكرورنا عبارة عن فئة تتحكم في معدل النسخ الجينى و تلعب دوراً هاماً في عدوى الالتهاب الكبد الوبائى و معدل إنتاج الانترفيرون. يهدف هذا العمل لإيجاد مقياس للتنبؤ للإستجابة للعلاج بالانترفيرون ألفا المبيجل و الريبافرين بإستخدام مير-٢٠a، مير-١٥٥، مير-٢١ ومير-١٩٦b بالإضافة لتقييم النسخ الجينى لMxA-mRNA كمؤشر إيجابى لعمل الانترفيرون. أجريت هذه الدراسة على مائة مريضاً(مستجيبين وغير مستجيبين للعلاج بالانترفيرون ألفا المبيجل والريبافرين) مصابين بفيروس سي وعشرون متطوعاً من الأصحاء. و لقد تم فصل الميكرورنا من مصل الدم و فصل MxA-mRNA من الدم و قياس مستوى النسخ بإستخدام تفاعل البلمرة المتسلسل الوقتى. أظهرت النتائج إرتفاع مستوى نسخ الميكرورنا الاربعة لغير المستجيبين للعلاج مقارنة بالمستجيبين للعلاج بالانترفيرون ألفا المبيجل و الريبافرين. كما تم تأكيد تلك النتائج عن طريق تثبيط مستوى نسخ MxA-mRNA لغير المستجيبين للعلاج مقارنة بالمستجيبين للعلاج. و لقد أظهرت تحليل النتائج عن طريق منحنى ROC أن القيمة الفاصلة للإستجابة للعلاج بالانترفيرون ألفا المبيجل و الريبافرين لمير-٢٠a، مير-١٥٥، مير-٢١ و مير-١٩٦b هى > ١، > ٢.٢٦، > ١.٥٧، > ٢.٤٩ علي التوالى. و نتيجة لذلك فإن تلك الميكرورنا (مير-٢٠a، مير-١٥٥،مير-٢١ و مير-١٩٦b) السابحة في مصل الدم تعد واحدة من المؤشرات الواعدة للإستجابة للعلاج بالانترفيرون ألفا المبيجل و الريبافرين للمرضى المصريين المصابين بفيروس سى.