

Analysis of Micro RNA 29, 122 and 155 Gene Expression in Immune Cells of Egyptian Patients with Chronic Hepatitis C Virus Infection

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ABSTRACT

Infected patients with chronic hepatitis C virus (HCV) showed high numbers of myeloid-derived suppressor cells (MDSCs) which are known to suppress the immune response. Given that microRNAs regulate the expression of many genes, it is important to test whether MDSCs correlate with altered miRNA expression. **Aim:** The present study aimed to assess the expression of miR-29, 122 and 155 in chronic HCV patients under interferon (IFN) therapy and their correlation with the frequency of MDSCs. A total of 28 subjects, including eighteen chronic HCV patients, and 10 healthy volunteers were enrolled in this study. MiR-29, 122 and 155 were evaluated using real-time- polymerase chain reaction (RT-PCR). Flow cytometric analysis was performed to evaluate the MDSCs which defined as Lin⁻HLA-DR⁻ CD33⁺ CD11b⁺. **Results:** miR-29 and 122 expressions in HCV patients showed significant decrease when compared to their control. However, there was an increase (but non-significance) in miR-155 in HCV patients than control subjects. Both relative and absolute numbers of MDSCs were increased in the peripheral blood in HCV patients as compared to control subjects. Moreover, non- significance change in IFN α / β receptors was observed in HCV patients as compared to control subjects. **Conclusion:** High numbers of MDSCs in chronic HCV patients correlate with low expression of miR-29 and 122.

Keywords: Micro RNA, gene expression, Egyptian patients, HCV, MDSCs.

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INTRODUCTION

HCV infection is a worthy cause of liver disease worldwide, most of the infected patients subjected to hepatocellular carcinoma (Khattab *et al.*, 2011). HCV persistence is due to its ability to escape from the adaptive and innate immune system components (Sklan *et al.*, 2009). The standard treatment for chronic HCV consisted of a combination of pegylated alpha interferon (IFN α) and ribavirin was in use till 2011. Recently, new generations of HCV

therapy such as sofosbuvir, daclatasvir, and ledipasvir have been approved (Sundaram *et al.*, 2016).

MicroRNAs were discovered in 1993 during a developmental timing experiment in the nematode *Caenorhabditis elegans* (Friedman *et al.*, 2009). miRNAs constitute a class of noncoding RNAs; about 18-22 nucleotides long and play a crucial role in the regulation of gene expression (Bartel, 2009). Many studies have identified several miRNAs as key players in

virus-host interactions, regulating virus replication and pathogenesis during HCV infection (Li *et al.*, 2011; Shrivastava *et al.*, 2013; Zhang *et al.*, 2012). miRNAs regulate the expression of many genes and subsequently control various cellular processes, such as the immune response to viral infections (El-Ekiaby *et al.*, 2012). As such these miRNAs play critical roles in HCV infection. Further, miRNAs regulate HCV life cycle either by supporting viral replication or by inhibiting IFN signaling pathways (Shrivastava *et al.*, 2013). For example, miR-122 is required for HCV replication in infected hepatocytes (Jangra *et al.*, 2010; Zhang *et al.*, 2012; Shrivastava *et al.*, 2013).

Further, in infected patients with HCV, miR-155 expression was markedly increased and promotes hepatocyte proliferation and tumorigenesis (Zhang *et al.*, 2012). It has found that liver hepatocytes of HCV patients expressed a low level of miR-29, by increasing its expression, the inhibition of viral RNA was reported (Bandyopadhyay *et al.*, 2011).

MDSCs represent a heterogeneous subset of immature myeloid cells derived from bone marrow that, under certain chronic conditions, including inflammation, cancer, and infection, do not differentiate into terminal cells such as granulocytes, macrophages, or dendritic cells (Ko *et al.*, 2009). Therefore, MDSCs are involved in the immune tolerance of various diseases, such as cancers, acute and chronic infection diseases (Wu *et al.*, 2014). In addition, MDSCs have been found to play a crucial part in suppressing the host's innate and adaptive immune responses (Younis *et al.*, 2016). We and others have reported that chronic HCV patients harbor high numbers of MDSCs in non-responders than in responders (Grutzner *et al.*, 2016; Younis *et al.*, 2016; Salem *et al.*, 2017). The responders have the tendency of lower MDSC levels than non-responders (Salem *et al.*, 2013).

The present study aimed to assess the expression of miR-29, 122 and 155 in IFN non-responders HCV patients and their correlation with the frequency of MDSCs. In conclusion,

high numbers of MDSCs in chronic HCV patients correlate with low expression of miR-29 and 122.

MATERIALS AND METHODS

Subjects

This study was conducted in accordance with guiding principles of the Declaration of Helsinki, approved by the Ethical Committee, Faculty of Medicine, Tanta University. Eighteen (18) patients with chronic HCV infection (age = 42 ± 4.5 years; male/female: 12/6) were recruited from The Tropical Medicine and Infectious Diseases Department, Tanta University (Tanta, Egypt). Ten volunteers subject (age = 35.6 ± 4.93 years; male/female: 6/4) were also recruited to compare the results.

Treatment of the patients

Patients enrolled in the present study treated with pegylated IFN α (long-acting interferon) once every week for 48 weeks plus daily treatment with 800–1200 mg ribavirin. The treatment period varied from patient to another.

Reagents and antibodies

BD FACS lysis buffer purchased from BD Bioscience (San Diego, CA, USA). Human monoclonal antibodies (hmAbs) purchased from eBioscience (San Diego, CA, USA): anti-Lin-1 (clone HI10a), anti- HLA-DR (clone L243), anti-CD33 (clone P67.6), anti-CD11b/MAC-1 (clone ICRF44) and anti-IFN α/β (clone 85228). RNeasy Mini Kit and Omniscript RT Kit (Qiagen, Germany). TaqMan probes used in this study were: 18S, hsa-miR 122, hsa-miR 155 and hsa-miR 29. All the probes were obtained from Applied Biosystems (Foster City, CA, USA) and they were used at concentrations recommended by their manufacturers.

Blood samples

Blood samples (10 mL) were collected by trained laboratory technicians in the clinic at Tropical Medicine and Infectious Diseases, Tanta University (Tanta, Egypt).

Total RNA extraction

Using the Omniscript extraction Kit, as per manufacturer's instructions. After RNA extraction, samples were kept at -80 °C till use.

Analysis of RNA and miRNA concentration with NanoDrop.

The concentration and purity of the large RNA (mRNA) fractions (> 200 nucleotides) was assessed using the NanoDrop spectrophotometer (NanoDrop Technologies). RNA concentration was automatically calculated using the formula: RNA concentration (ng/μL) = (A260 x e)/b, where: A260 = Absorbance at 260 nm, e = extinction coefficient (ng-cm/ml), b = path length (cm) RNA with an absorbance ratio at 260 and 280 nm (A260/A280) between 1.8 and 2.2 were indicative of pure RNA.

Reverse transcriptase (cDNA synthesis) and RT-PCR analysis.

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 5 ng of RNA using the Omniscript RT Kit (Qiagen), as per manufacturer's instructions. Gene expression was measured using quantitative real-time PCR and TaqMan probes (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 20 μL. Ribosomal 18s RNA was used as the internal standard. RT-PCR was performed on a Step One real-time PCR system (Applied Biosystems). RT-PCR System with the following conditions: 95°C for 10 min, followed by 40 cycles at 94°C for 15 s, 55°C for 30 s, 70°C for 35 s, post extension 72°C for 10 min and hold at 4°C. The relative quantification of the target transcripts normalized to the endogenous control was determined by the comparative cycle threshold (CT) method. Relative changes in gene expression between samples were analyzed using the $2^{-\Delta\Delta(Ct)}$ method (Barshack *et al.*, 2010).

MDSCs and IFNα/β assay by Flow Cytometry

Staining with mAbs done on blood. Briefly, 100 μL whole blood, stained with the indicated hmAbs using the concentrations recommended by the manufacturers. FITC, PE, Cychrome and APC-coupled antibodies added to the samples for 20–30 min in dark and then the BD FACS buffer added for lysis of red blood cells. Then, the cells washed twice by using phosphate buffer saline (PBS). Acquisitions performed with a CyFlow Cube 8- Partec flow cytometer (Partec GmbH, Münster, Germany) and data analysis were done by FlowJo software. For determination of MDSCs population, the phenotype of MDSCs in whole blood was analyzed using flow cytometric analysis. Different gating strategies were used to determine the circulating MDSCs levels in the peripheral blood. After staining blood with anti-HLA-DR, anti-CD11b, anti-CD33, and anti-LIN, assessed in different patients in different weeks. The MDSCs population was defined as Lin⁻ HLA-DR⁻ CD33⁺ CD11b⁺. For determination of IFNα/β receptor, peripheral blood cells were processed with anti-IFN α/β.

Statistical analysis

Data were expressed as the mean ± standard error (SE) and were analyzed using SPSS software version 16.0. Various assay conditions were evaluated using non-parametric Tests (Chi-square Test) and (Wilcoxon Signed Ranks Test and Mann-Whitney Test for gen data analysis). Analysis of variance [ANOVA] test was used for comparison among various times in the same group in quantitative data. The level of significance was accepted when ($P < 0.05$).

RESULTS

Subjects demographics.

There was no significant difference ($p = 0.415$) between the studied groups as regard to sex distribution according to chi-square test. No significant difference ($p = 0.167$) based on age between the studied groups according to a chi-square test (Table 1).

Table 1: Subjects demographics.

Parameter		Control	HCV patients
Age		35.6±4.9	43.9±2.3
Sex	Male	(60%)	(66.7%)
	Female	(40%)	(33.3%)

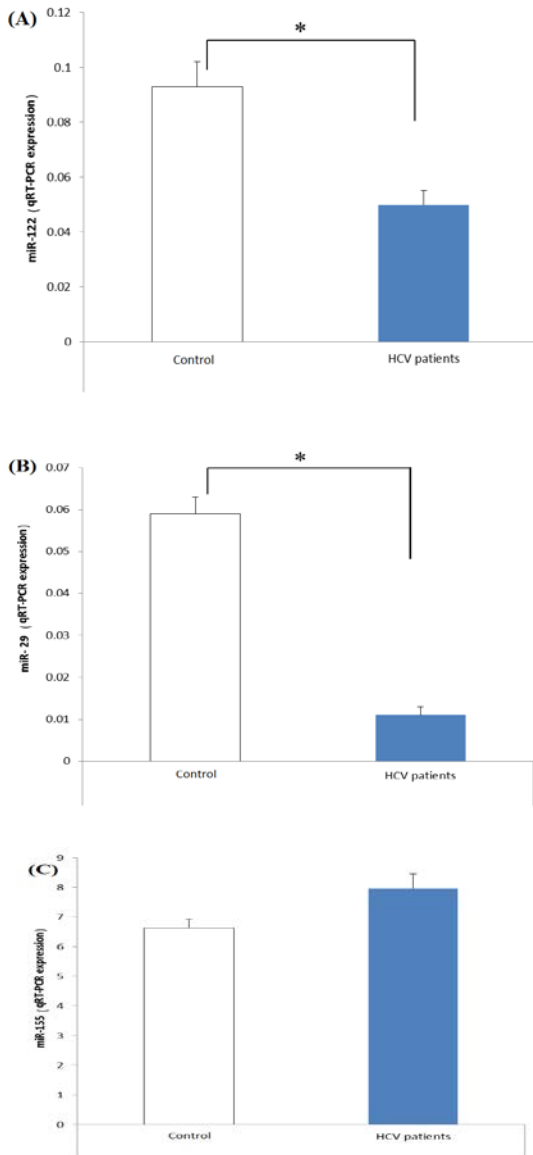


Figure 1: MiR-29, 122 and 155 expressions in control subjects and HCV patients. (A) Shows a decrease in miR-122 gene expression in HCV patients by 2-fold than control. (B) Shows a decrease in miR-29 gene expression in HCV patients by 5-fold than control. (C) Shows non- significance in miR-155 gene expression* $P < 0.05$.

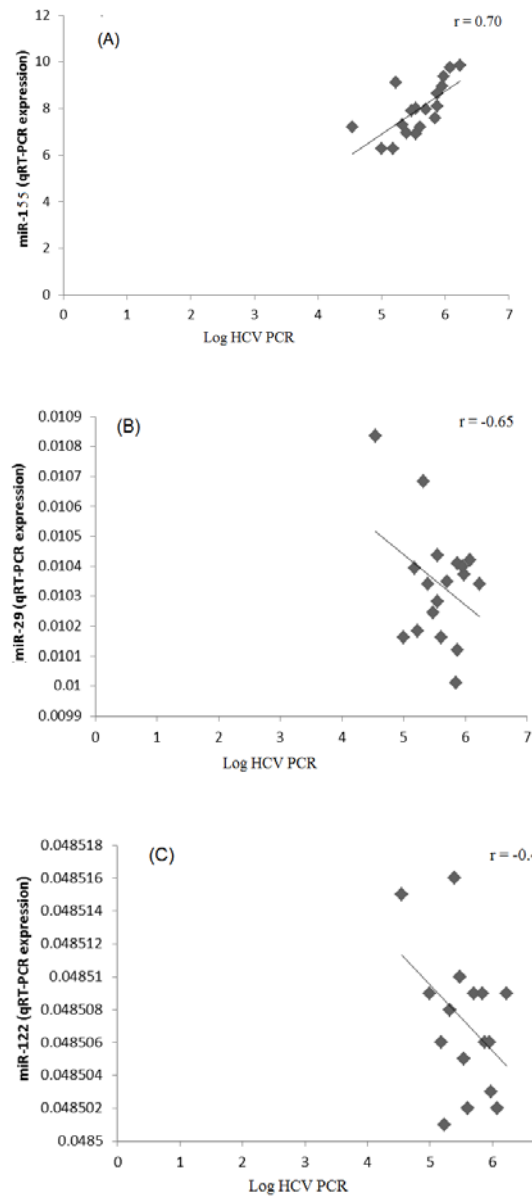


Figure 2: Correlation between miR-29, 122, 155 and log of viral load. (A) Shows a positive correlation between log HCV PCR and miR-155. (B and C) Show an inverse correlation between log HCV PCR and miR-29 and 122

Micro RNA gene expression in HCV patients and control subjects.

A significant decrease ($P < 0.05$) in miR-122 and 29 was demonstrated in HCV patients when compared with control subjects (Fig.1). However, there was an increase (but non-significance) in miR-155 in HCV patients than control subjects (Fig.1). MiR-29 decreased by 5-fold and miR-122 by 2-fold in patients than control.

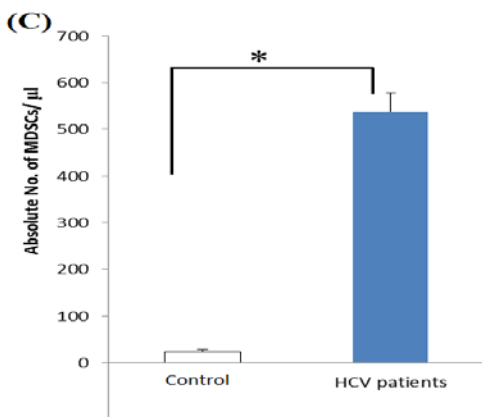
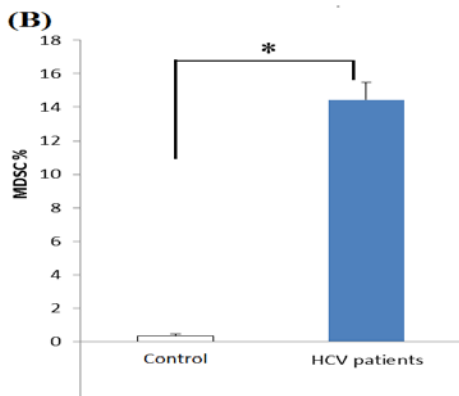
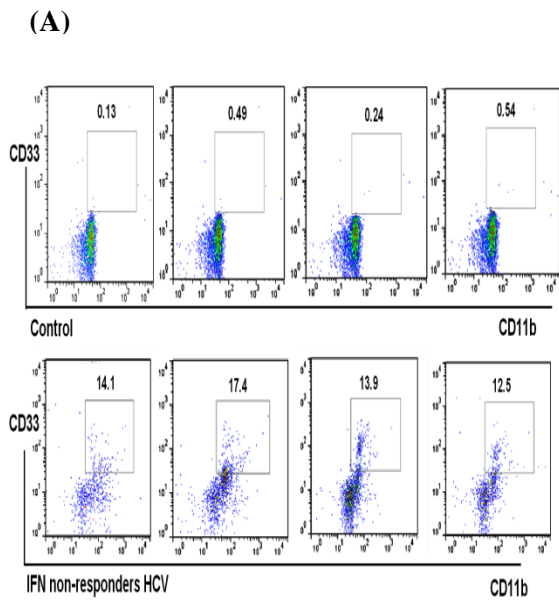


Figure 3: (A) Representative flow cytometry analyses of MDSCs from control subjects and HCV patient's samples. (B) Relative and (C) absolute numbers of MDSCs in HCV patients compared to control.

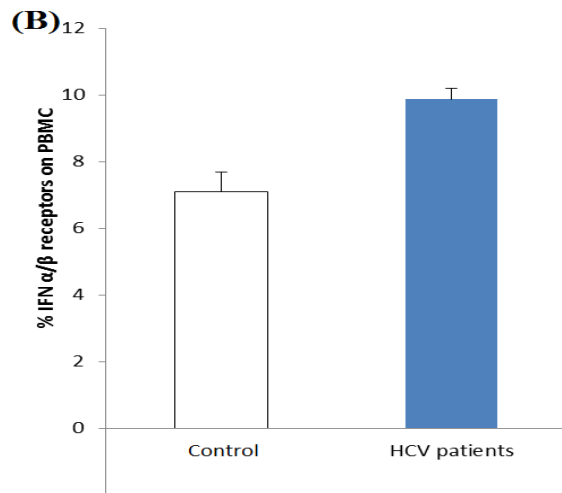
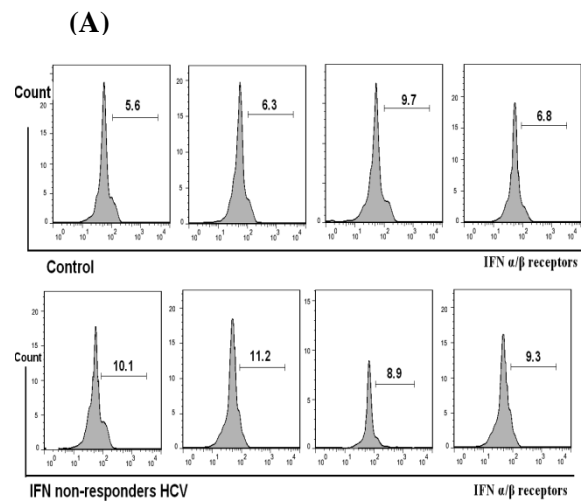


Figure 4: IFNα/β receptors in control and HCV patients. (A) Shows Flow cytometry analyzes of IFNα/β receptors was gated from RBC-lysed fresh blood from control and HCV patients. (B) Shows relative numbers of IFNα/β in HCV patients compared to control. c: control; p: patient.

Correlation between miR-29, miR-122, miR-155 and viral load

To further verify the correlation between the expression levels of miR-122, 155 and miR-29 with viral load among the HCV cases, multivariate logistic regression analysis was performed using Wilcoxon Signed Ranks Test. As shown in Fig. 2 and Table 2, log HCV PCR showed a significant inverse correlation with miR-122 and miR-29 quantitative expression levels in HCV patients ($P < 0.05$).

Table 2: Viral titer for all patients.

No. of patient	No. of week	Log HCV- RNA (mean \pm SE)
2	20	5.9 \pm 0.06
3	24	6.0 \pm 0.09
3	28	5.8 \pm 0.05
4	32	5.5 \pm 0.04
2	36	5.3 \pm 0.04
3	40	5.2 \pm 0.04
1	44	4.5 \pm 0.03

No.: number and SE: standard error

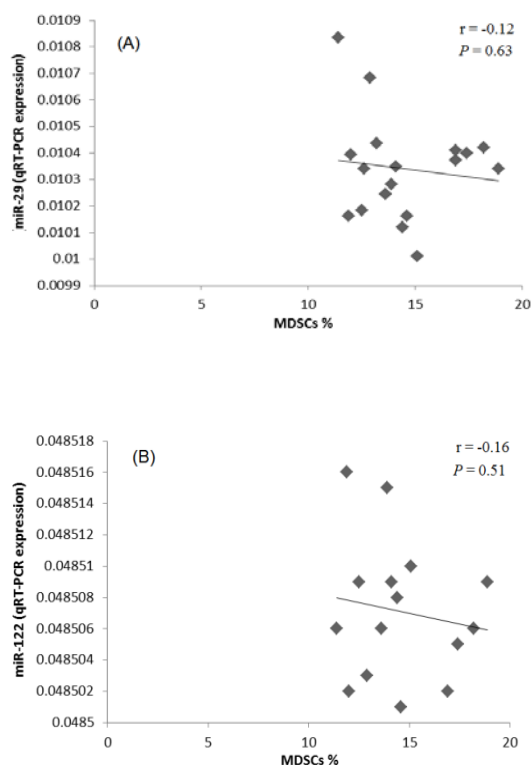


Figure 5: Correlation between miR-29, 122 and MDSCs % in HCV patients. Show inverse correlation between miR-122 (A), 29 (B) and MDSCs (%).

Numbers frequency of MDSCs

The numbers of MDSCs were higher in the blood of chronic HCV patients than control subjects (Fig. 3). However, there was an increase (but non-significance) in IFN α / β receptors expression in HCV patients than control subjects (Fig. 4).

Correlation of MDSCs, miR-122, 155 and miR-29 in chronic HCV patients.

Multivariate logistic regression analysis was done using the Wilcoxon Signed Ranks Test. MDSCs % showed a significant inverse correlation ($P < 0.05$) with miR-29 and miR-122 expression (Fig. 5).

DISCUSSION

MDSCs and miRNAs contribute to attenuating immune responses during chronic viral infection; however, the precise mechanisms underlying their suppressive activities remain incompletely understood (Ren *et al.*, 2016). MicroRNAs represent an interesting field of investigation with regards HCV infection and replication. Furthermore, they may represent new targets for the development of antiviral therapeutics (Ramachandran *et al.*, 2013). Our data suggested that high numbers of MDSC and expression of miR-155 with low expression of miR-122 and 29 inhibit the response of IFN treatment. Down-expression of miR-122 after HCV infection directly promote HCV replication, translation, and its IFN therapy antagonist (Marquez *et al.*, 2010). Zhang *et al.*, (2012) were reported that miR-122 positively modulates HCV infection through direct interactions with viral RNA and stimulates HCV translation. Patients with low pretreatment miR-122 levels are less likely to achieve viral clearance through PEG-IFN- α plus ribavirin combination therapy (Sarasin-Filipowicz *et al.*, 2009). HCV infected patients who did not respond to therapy had significantly lower miR-122 levels as compared to IFN responder HCV (Sarasin-Filipowicz *et al.*, 2009). However, in chronically infected patients, hepatic miR-122 level decreased (Choi *et al.*, 2013). MiR-29 was down-regulated in most HCV-infected patients. MiR-29 regulates the expression of extracellular matrix proteins (Bandyopadhyay *et al.*, 2011). Patients with advanced liver cirrhosis showed the significantly low level of miR-29 in their serum when compared with healthy controls (Roderburg *et al.*, 2011). MiR-29 has been shown to induce myeloid progenitor expansion. It is possible that aberrant expression of miRNAs contributes to MDSC expansion under pathological conditions (Ren *et al.*, 2016).

In contrast, there was more increase in miR-155 in IFN non-responders HCV patients than

control (Bandyopadhyay *et al.*, 2011). Increased expression of miR-155 in serum, peripheral mononuclear cells, and liver tissues of patients with chronic HCV infection could be a negative prognostic marker. Expression of miR-155 did not differ between HCV infected and control PBMCs (El-Ekiaby *et al.*, 2012; Zhang *et al.*, 2012). Treatment-naïve patients with chronic HCV infection have been shown to have higher expression of miR-155 in their circulating monocytes as compared to individuals who cleared HCV infection after therapy, suggesting a correlation between increased miR-155 and HCV viral presence and/or replication (Bala *et al.*, 2012).

High numbers of MDSCs were detected in chronic HCV patients as compared to control subjects (Salem *et al.*, 2017). The percentage of MDSCs increased in chronic HCV infected patients and might be related to persistent HCV infection (Liu *et al.*, 2014). Salem *et al.*, (2013) reported that chronic HCV patients showed elevated levels of MDSCs regardless of IFN therapy. The responders have the tendency of lower MDSC levels than non-responders. Ning *et al.*, (2015) were founded that the frequency of M-MDSCs from HCV infected patients was significantly increased when compared to healthy controls. Our data showed that MDSC has a positive correlation with miR-122 and 29 in control but has a negative correlation in patients. MiR-29 was significantly decreased in CD33⁺ myeloid cells from HCV-infected patients compared with healthy participants, revealing a decline in miR-29 expression in myeloid cells in the setting of chronic HCV infection lead to inhibition STAT-3 (Ren *et al.*, 2016).

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