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Changes in the co-expressions of interleukin 29 (IL-29), IFN-inducible protein 10 (IP-10) and monokine induced by IFNγ (MIG) genes in chronic hepatitis C Egyptian patients untreated and treated with DAAs

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Summary.– Direct acting antiviral agents (DAAs) are a group of antiviral drugs that inhibit specific non-structural proteins of the virus and disrupt viral replication and infection. DAAs regimens for hepatitis C virus (HCV) infection provide a particular event to tackle mechanistic intracellular relationships between the innate immunity and HCV, potentially providing perceptions about the rate of the viral replication and complex decay. Interleukin 29 (IL-29) prevents the replication of HCV. IFN-inducible protein 10 (IP-10) plays a significant role in the pathogenesis of HCV infection. MIG/CXCL9 are produced by inflammatory and stromal cells such as hepatocytes following either stimulation by interferon lambda (IFNγ) or viral infection. This study aimed to evaluate the co-expression of IL-29, IP-10 and MIG in peripheral blood mononuclear cells (PBMCs) from untreated and treated chronic HCV patients with DAAs. This study included group of twenty naïve HCV patients, group of twenty sustained viral response (SVR) patients and a control group that consisted of 10 healthy subjects. All subjects were tested for liver enzymes, serum albumin level, total serum bilirubin, platelet count, prothrombin activity and viral load. Relative gene expression of IL-29, IP-10, and MIG in PBMCs from all subjects was determined using real time PCR. The mean value of IL-29, IP-10 and MIG gene expression significantly increased in both naïve HCV and SVR groups of patients as compared to normal subjects. The corresponding value was significantly lower in patients with SVR compared to naïve HCV patients. Infection with HCV significantly trigged the co-expression of IL-29, IP-10, and CXCL9 (MIG) genes in PBMCs of chronic hepatitis C patients and significantly down-regulated in those who achieved SVR after successful DAAs therapy.

Keywords: IP10; MIG; IL29; HCV; DAAs; gene expression

Introduction

Globally, an estimated 170 million people have chronic hepatitis C infection. Approximately 399,000 people die yearly from hepatitis C, mainly due to cirrhosis and hepatocellular carcinoma (HCC)(Crowell, 2020). Hepatitis C viral infection in Egypt is endemic and has the highest prevalence rate worldwide (14.7%) (Elgharably *et al.*, 2017).

Treatment of chronic HCV infection has 2 targets: the first is the sustained eradication of the virus; the second one is to inhibit the disease progression into liver cirrhosis, HCC and decompensated liver requiring liver transplantation (Lavanchy, 2011).

The treatment of HCV has developed over the years when switching from interferon to direct acting antiviral agents (DAAs). DAAs are a group of antiviral drugs that

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Abbreviations: DC = dendritic cells; CHC = chronic hepatitis C; DAAs = direct acting antiviral agents; HCC = hepatocellular carcinoma; HCV = hepatitis C virus; IFN = interferon; IP-10 = IFN-inducible protein 10; IL = interleukin; PBMCs = peripheral blood mononuclear cells; SVR = sustained viral response

inhibit specific non-structural proteins of the virus and disrupt viral replication and infection (European Association for the Study of the Liver. Electronic address, 2017).

Interferon lambda (IFNλ) is an innate cytokine that was identified early in the last decade and classified as a new type III IFN. The IFNλ gene family is composed of three distinct genes: IFNλ1 (IL29), IFNλ2 (IL28A), and IFNλ3 (IL28B) (Sheppard *et al.*, 2003). The novel type III IFNs, such as IFN lambda 4 (IFNL4), was discovered about ten years later (Hamming *et al.*, 2013). The members of this IFN family interact with its unique receptors that differ from type I (IFNα/β) and type II (IFNγ) IFN cell receptors (Witte *et al.*, 2009).

Iinterestingly, hepatocytes of liver biopsy specimens from chronic hepatitis C (CHC) patients have higher IFNλ receptor expression, while no expression is detected in fibroblasts, endothelial cells, adipocytes, or primary central nervous system cells (Pagliaccetti and Robek, 2010).

Interleukin 29 (IL-29) can be induced in hepatocytes and other liver cell types, particularly in Kupffer cells, stellate cells, and dendritic cells (DC) which may be considered its main producers (Bolen *et al.*, 2014). During CHC, the IL-29 immune response can only control HCV replication but not completely eliminate the virus. This is partially due to viral mechanisms counteracting the immune response (Ferreira *et al.*, 2016; Hermant and Michiels, 2014).

IFN-inducible protein 10 (IP-10) is a CXC chemokine produced by peripheral blood mononuclear cells (PBMCs), fibroblasts, and endothelial cells, which play an important role in the maintenance of chronic inflammatory responses by promoting the recruitment of monocytes, T cells, and NK cells into the target tissue and/or organs (Tacke *et al.*, 2011). It has been suggested that IP-10 plays an important role in chronic HCV infection by directing T-helper 1 (Th1) lymphocyte recruitment to the liver. This immune reaction results in progressive liver damage (Cavalcante and Lyra, 2015).

The CXC chemokine family is a family of pleiotropic molecules. They participate in the trafficking of various leukocyte subsets, vascular remodeling, and angiogenesis (Park *et al.*, 2001). Monokine induced by IFNγ (MIG/CXCL9) is produced by monocytes, macrophages, neutrophils, and other inflammatory cells, and by stromal cells such as hepatocytes, following either stimulation by IFNγ or viral infection (Shields *et al.*, 1999). It has been found that MIG expression occurs on sinusoidal epithelial cells in both normal and HCV-infected liver tissue, and expression is up-regulated in the latter tissue (Zeremski *et al.*, 2007). Both peripheral and intrahepatic expression levels of these chemokines are elevated in HCV-infected patients with a high degree of inflammation and fibrosis (Zeremski *et al.*, 2009, 2008).

The aim of this study was to evaluate the co-expression of IL-29, IP-10 and CXCL9 (MIG) genes in PBMCs from chronic HCV patients untreated and treated with DAAs compared to corresponding expression in healthy control subjects.

Materials and Methods

Subjects. This study included 40 chronic HCV Egyptian patients divided into two groups. The naïve HCV group consisted of 20 patients who did not receive therapy and sustained viral response (SVR) group consisted of another 20 patients who fulfill the criteria for the national protocol of treatment of CHC and who achieved successful SVR after DAAs regimen composed of Sofosbuvir 400 mg + Daclatasvir 60 mg + Ribavirin 1000–1200 mg obtained daily for twelve weeks. Additionally, a third group (control group) consisted of 10 healthy subjects, with matching age and sex (Table 2). Patients with hepatitis A, hepatitis B, and/or HIV infections were excluded from the study. Patients suffering from malignancies or any immunological disorders were not included. Chronic HCV patients were selected from the Hepatology Department, Medical Research Institute, University of Alexandria. All relevant information was collected from all subjects under study including personal data as age, gender, residence, smoking and alcohol consumption, as well as health data (history of blood transfusion, previous surgical interference, dentistry, history of hepatic encephalopathy and jaundice). Also, clinical and ultrasound examinations of the abdomen were done to assess the presence of ascites or HCC. The work was completed according to the ethical rules of the Medical Research Institute, University of Alexandria, Egypt and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Sampling. After signing a written consent, all patients were subjected to blood withdrawal to obtain heparinized, citrated, and EDTA treated blood and sera samples.

Routine laboratory investigations. All subjects were tested for liver enzymes alanine aminotransferase (ALT) and aspartate transaminase (AST), serum albumin level, total serum bilirubin, platelet count, and prothrombin activity.

Detection of HCV antibodies. For the laboratory diagnosis of HCV infection, Murex anti-HCV version 4 (Diasorin, France) was used according to the manufacturer's instructions.

Estimation of the HCV viral load. For confirmation of persistent chronic infection in group I and SVR in group II, HCV viral RNA was quantified in patients' sera. Viral RNA was extracted from patients' sera via QIAamp viral RNA mini spin kit (Qiagen®, Germany) according to the manufacturer's instructions. The extracted viral RNA was used as a template for viral load determination using Artus HCV QS-RGQ-PCR kit (Qiagen®) and real time PCR machine MX3000P ™ (Stratagene, USA).

Table 1. Sequences of primer pairs used for real-time PCR amplification

Gene	Primer	Sequence (5'–3')	Reference
IL-29	IL29-F	TAT CCA GCC TCA GCC CAC AG	Wu *et al.*, 2011
	IL29-R	CTC AGA CAC AGG TTC CCA TCG	
MIG	MIG-F	TGC AAG GAA CCC CAG TAG TGA	Okamoto *et al.*, 2008
	MIG-R	GGT GGA TAG TCC CTT GGT TGG	
IP-10	IP10-F	TGA AAT TAT TCC TGC AAG CCA A	Pu and Wang, 2014
	IP10-R	G ACA TCT CTT CTC ACC CTT CTTT	
β-actin	Act-F	CAC TCT TCC AGC CTT CCT TCC	Wu *et al.*, 2011
	Act-R	AGG TCT TTG CGG ATG TCC AC	

Table 2. Demographic and laboratory data of the studied groups

Parameter	Naïve HCV group (n = 20)	SVR group (n = 20)	Control group (n = 10)	P-value
Age (years) range mean±SD	 26–56 45±8	 36–63 44±8.4	 28–60 43±12	 0.674
Male/ Female	12/8	14/6	6/4	0.64
AST (U/L) range mean±SD	 25-65* 37.5±96	 20–51* 32.1±9.1	 9–22 16.1±4.6	 0.00
ALT(U/L) range mean±SD	 21–73* 37.2±13.2	 23–45* 34.1±7.6	 9–20 14.7±3.7	 0.00
Platelet count×10^3 range mean±SD	 109–348* 191±70	 136–327* 195.4±56	 250–431 335.3±58	 0.00
PT activity range mean±SD	 70–90%* 79.8±6.9	 70–84%* 87.8±4.5	 90–100% 95.8±3.4	 0.00
Serum albumin(g/dl) range mean±SD	 3.3–4.0* 3.7±0.25	 3.4–4.3* 3.8±0.35	 3.8–4.7 4.17±0.28	 0.002
Total bilirubin (mg/dl) range mean±SD	 0.5–1.85* 0.9±0.34	 0.6–1.2* 0.96±0.15	 0.4–1.0 0.71±0.1	 0.04
Viral load (IU/ml) range mean±SD	 65,533–2,921,707 579,623±738147.8	below detection limit	 –	

*Statistically significant at p <0.5 as compared to control group.

HCV genotyping. Keeping the fact that more than 90% of HCV genotype among Egyptian patients is HCV-4 (Ray *et al.*, 2000; Wantuck *et al.*, 2014), HCV genotyping is currently not a part of the national program for the treatment of chronic HCV infection in Egypt. Patients who failed to achieve SVR are subjected to HCV genotyping.

Isolation of PBMCs. Peripheral blood mononuclear cells were isolated from heparinized blood using Ficoll gradients within 2–4 h from blood withdrawal. Cells were counted on a modified Neubauer hemocytometer and adjusted to 1x10^6 cells/ml. Cells were subjected directly to RNA extraction or stored at -80°C until needed.

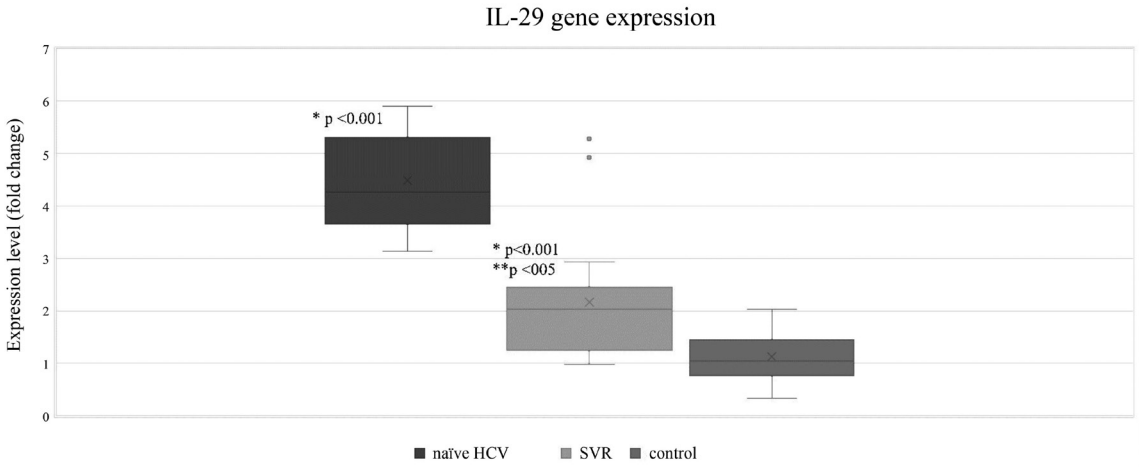

Fig. 1

IL-29 gene expression in PBMCs
The mean value (±SD) of IL-29 gene expression in PBMCs was 4.486±0.918 and 2.166±1.168 in naïve HCV and SVR patients, respectively. The mean value of IL-29 gene expression was significantly increased in both naïve HCV and SVR groups of patients as compared to healthy subjects. The corresponding value was significantly decreased in patients with SVR as compared to naïve HCV patients. SVR: Sustained viral response. *: Significant difference in relation to healthy patients. **: Significant difference in relation to naïve HCV patients. X represents the mean.

RNA extraction and cDNA synthesis. Total RNA was extracted using PurLink RNA mini kit (Invitrogen, USA) according to manufacturer instructions. RNA concentration and purity were estimated on Nanodrop 2000 (Thermo Scientific, USA). A total of 1 µg of RNA was reverse transcribed using High-Capacity cDNA reverse transcription kit (Applied Biosystems, USA) and random hexamer primers.

Gene expression. Real-time PCR quantitation of IL-29, IP-10, MIG, and the housekeeping gene β-actin (Table 1) was performed in duplicates using SYBR green master mix. The PCR reaction composed of 10 µl Maxima SYBR Green qPCR master mix (Thermo Scientific, USA), 0.2 µM of forward, and reverse primers and 2 µl cDNA. PCR was performed on Stratagene MX 3000P using the thermal profile 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. PCR products were subjected to melting curve analysis to confirm the efficiency of amplification. The relative gene expression was calculated using ΔΔCT method. All CT values were normalized against CT value of β-actin and gene expression in test groups was compared relative to the control healthy group.

Statistical analyses. Data were fed to the computer and analyzed using IBM SPSS software package version 23.0. Quantitative data were expressed using range (minimum and maximum) mean ± standard deviation. For normally distributed data, comparisons between the different studied groups were done using F-test (ANOVA) and Post Hoc test (LSD) for pairwise comparison. Correlations between two quantitative variables were assessed using Pearson or Spearman coefficients regarding the normality of the data. The significance of the results was judged at the 5% certainty level. Statistical significance of the difference between studied groups regarding the degree of gene expression was calculated using manually applied Post Hoc test (LSD) test. The final level of significance was calculated using IBM SPSS computerized system.

Results and Discussion

CHC infection is associated with changes in both adaptive and innate immune systems that lead to viral persistence. DAA therapy shows high efficacy in eradicating viral infection. Nevertheless, DAAs do not directly trigger cellular or humoral immune response against infection. Therefore, analysis of changes in the expression of immune markers involved in CHC pathogenesis after successful therapy provide insights on the immune response to HCV treatment with DAAs.

The study considers the evaluation of the co-incident gene expression of IL-29, IP-10, and MIG in PBMCs from patients with chronic HCV infection. The current study was conducted on 20 untreated chronic HCV patients, 20 treated chronic HCV patients showing SVR after DAAs treatment, and 10 normal healthy individuals.

As expected the serum level of aminotransferases were significantly higher in chronic HCV patients than in healthy control subjects, while there was no significant difference between the two chronic HCV groups (naïve and SVR) regarding those parameters. In accordance

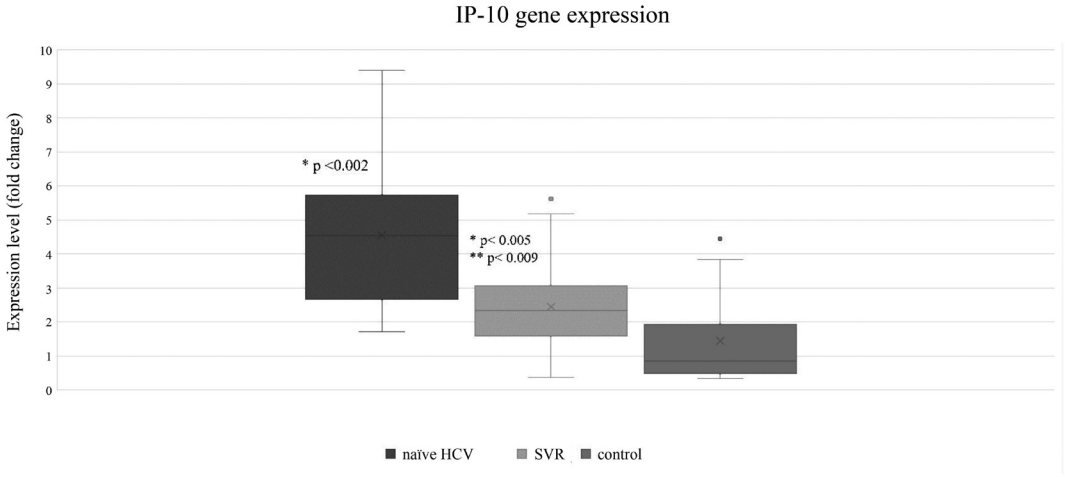

Fig. 2

IP-10 gene expression in PBMCs

The mean value (±SD) of IP-10 gene expression in PBMCs was 4.544±1.99 and 2.4416±1.258 in naïve HCV and SVR patients, respectively. The mean value of gene expression in the healthy patients group is considered equal to 1. The mean value of IP-10 gene expression was significantly increased in both naïve HCV and SVR groups of patients as compared to normal subjects. The corresponding value was significantly decreased in patients with SVR as compared to naïve HCV patients. SVR: Sustained viral response. *: Significant difference in relation to healthy patients. **: Significant difference in relation to naïve HCV patients. X represents the mean.

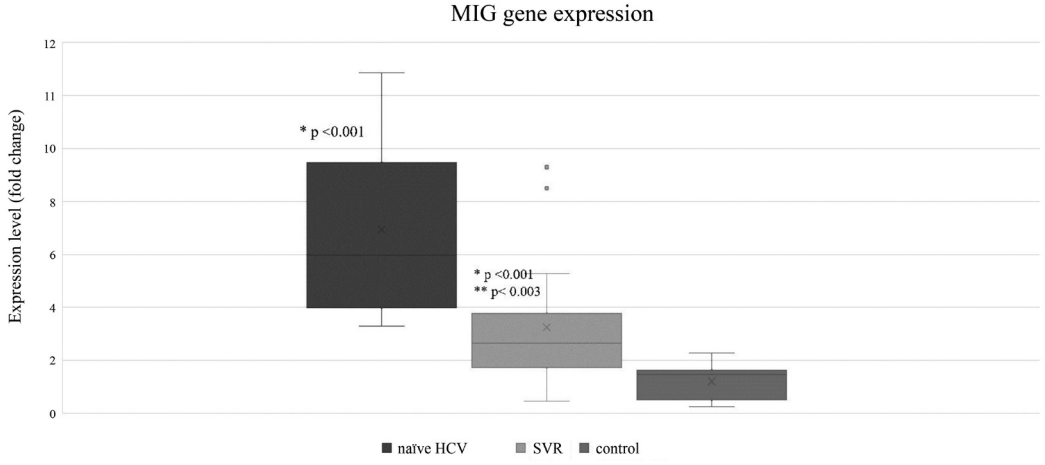

Fig. 3

MIG gene expression in PBMCs

The mean value (±SD) of MIG gene expression in PBMCs was 6.93±3.14 and 3.2±2.2872 in naïve HCV and SVR groups, respectively. The mean values of MIG gene expression were significantly increased in both naïve HCV and SVR groups of patients as compared to control subjects. The corresponding value was also significantly decreased in the SVR group as compared to the naïve HCV group. SVR: Sustained viral response. *: Significant difference in relation to healthy patients. **: Significant difference in relation to naïve HCV patients. X represents the mean.

with (Wu *et al.*, 2016) reporting that the serum level of aminotransferases, either normal or elevated, was not associated with SVR rate. In laboratory investigation results, platelet count, activity and total bilirubin were significantly lower when compared to the control group.

Our results provide a proof for the significant induction of IL-29 gene expression in PBMCs from chronic HCV patients.

The large number of researches conducted on IFNλs has led to several inconsistencies, and controversies. Mihm

et al. (2004) showed no difference between HCV diseased liver and non-viral diseased liver during the analysis of IFNλ in liver biopsies. On the other hand, Diegelmann *et al.* (2010), showed up-regulated serum levels of IFNλ in chronic HCV patients when compared to serum levels of patients with either non-viral diseased livers or control non-diseased livers. On the contrary, Langhans *et al.* (2011) have shown lower IFNλ serum levels in chronic HCV livers compared to non-diseased livers.

Dolganiuc *et al.* (2012) reported increased levels of IFNλ protein in sera and mRNA in livers of chronic HCV patients, but not in those with HCV who achieved sustained viral response.

Yoshio *et al.* (2013) showed that blood dendritic cell antigen 3, (BDCA3(+) DCs, mDCs2) recovered from PBMC, or the liver of chronic HCV patients released significant levels of IFNλs when stimulated with cell-cultured HCV or HCV-transfected Huh7.5.1. cell line (Huh7.5). This is in agreement with Alborzi *et al.* (2015) results, who reported that IL-29 serum level of HCV infected patients is elevated compared to those of healthy individuals. Increased expression of IL-29 in chronic HCV patients before treatment may be attributed to direct induction by endogenous IFNα and IFNγ activation of DCs with increased TLR-3 (Broering *et al.*, 2014; Zhang *et al.*, 2013).

The less pronounced increase of IL-29 expression in chronic HCV with SVR after treatment with DAAs may be due to the high success rate of DAAs with complete clearance of the HCV virions from the hepatocytes and loss of triggering factor of the immune system (Bruening *et al.*, 2017).

Regarding IP-10, current results proved the significant induction of IP-10 gene expression in PBMCs from CHC patients and patients with SVR than in normal control subjects.

Zeremski *et al.* (2008) found elevated intrahepatic mRNA levels of all CXC chemokines, most markedly CXCL10 (IP-10), in HCV-infected patients with higher necro-inflammation and fibrosis. Most intrahepatic lymphocytes express the CXCR3 receptor, and the number of CXCR3+ lymphocytes was increased in patients with advanced necro-inflammation.

Wan *et al.* (2009) reported that MIG and IP-10 were up-regulated transiently in patients with SVR before IFNα based treatment and in second week of treatment, indicating that these cytokines may correlate with viral clearance.

Li *et al.* (2012) reported that TLR3 recognizes HCV infection in cultured hepatoma cells, leading to NF-κβ activation and the production of numerous chemokines and inflammatory cytokines, such as RANTES, MIP-1α, MIP-1β, IP-10, and IL-6. Mutations specifically disturbing the dsRNA-binding activity of TLR3 diminished the chemokine/cytokine response to HCV infection, indicating that HCV dsRNA was the PAMP triggering TLR3 signaling. Additionally, robust secretion of chemokines and inflammatory cytokines was also detected in primary human hepatocytes after stimulation with extracellular poly-I:C, a TLR3 ligand.

Increased expression of IP-10 in CHC naïve patients in comparison with SVR patients may be attributed to the increase of TLR-3 in the first group (Li *et al.*, 2012). On the other hand, potent oral DAA therapy is associated with a rapid reduction in plasma IP-10 levels that is parallel with the reduction of HCV-RNA (Lin *et al.*, 2014).

Regarding the expression of MIG, Zeremski *et al.* (2008) found elevated intrahepatic mRNA expression of all three CXC chemokines in HCV-infected patients compared to non-infected subjects. Most of the intrahepatic lymphocytic cells express the CXCR3 receptor. Besides, the mean serum chemokine levels are significantly increased in HCV-infected patients compared to uninfected controls. Increased serum level of CXCL9 in CHC patients was also proved by Moura *et al.* (2010). More recently, Tsuge *et al.* (2011) proved that CXC chemokines maintained high IFN responsiveness under HCV infection.

Wandrer *et al.* (2016) found that PBMCs from patients with HCV infection who showed SVR to IFNα treatment up-regulated the expression CXCL9 and CXCL10, much more strongly than cells from patients with antiviral treatment failure. As a possible mechanism of the stronger IFNα-induced cytokine response, they detected elevated expression and higher phosphorylation level of the transcription factor STAT1 in PBMCs from patients with SVR. This is in contrast to our results as we found decreased expression of MIG in the SVR group than in untreated CHC. This contradiction in results may be due to the use of DAAs in the treatment of our CHC patients.

In partial agreement with our results, elevation in CXC chemokines was observed by Butera *et al.* (2005) in all patients with HCV. They showed that the expression level of CXCR3 was significantly up-regulated on peripheral blood B lymphocytes, but not T lymphocytes, from individuals with HCV infection. However, levels of CXCL10 and CXCL9 were down-regulated after successful antiviral therapy.

From our results, we can conclude that infection with HCV significantly triggers the co-expression of IL-29, IP-10 and CXCL9 (MIG) genes in PBMCs in chronic hepatitis C patients, while these genes were significantly down-regulated in those who achieved SVR after successful DAAs therapy.

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