Insights into the role of HCV Plus-/Minus strand RNA, IFN-γ and IL-29 in relapse outcome in patients infected with HCV

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Summary

Background and Objectives: **Approximately one-third of hepatitis C virus (HCV) infected patients who complete antiviral therapy with undetectable serum HCV RNA at the end of therapy (ETR), will experience relapse. The reasons for the failure of treatment have not been elucidated. It was showed that HCV RNA can persist and replicate in extra hepatic sites, e.g. in peripheral blood mononuclear cells (PBMCs), but the relevance of its presence with relapse over time is still unknown. Moreover, interferon-gamma (IFN-γ) and IFN-lambdas [IFN-λ1, interleukin-29 (IL-29)], possess potent antiviral activity. We studied if the presence of plus-/minus strand RNA in PBMCs of patients and the serum level of IFN-γ and IL-29, which is the most abundant IFN-lambdas in serum, can be considered as predictive factors in relapse outcomes.**

Methods: **Patients were screened for plus-/minus strand RNA at ETR and after 6 months. Also, we measured the serum level of IFN-γ and IL-29 and compared the result with those who developed a sustained virological response (SVR).**

Results: **Levels of IL-29 and IFN-γ serum were significantly higher in SVR at ETR and 6 months later compared to those of the relapsed patients, but there was no difference between the two groups regarding the presence or absence of plus-/minus HCV strand in PBMCs.**

Conclusions: **Our novel findings showed that the serum level of IL-29 and IFN-γ are predictive of relapse outcomes to HCV treatment, but there was no association between the presence of plus-/minus HCV RNA in PBMCs of patients with an outcome of therapy at ETR and later.** ***(Asian Pac J Allergy Immunol 2015;33:173-81)***

Keywords: *IL-29, IFN-γ, Relapsed, HCV*

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Introduction

Endogenous interferon (IFN) specifically IFNα, play a pivotal role in host defense against a viral infection and has been one of the therapeutic options for the treatment of chronic hepatitis C virus (HCV) infection.[1] For years, treatment with pegylated IFN (PEG-IFN)-α plus ribavirin, combination therapy has been the standard of care for patients with chronic hepatitis C (CHC) fostering sustained virological response (SVR) in nearly half of the patients with HCV infection.[2]

The mechanisms of virus elimination by antiviral therapy remain unclear. Several reports showed that in addition of viral factors (HCV genotypes and viral load), a number of baseline host-related factors (i.e. age, gender, race, body mass index, pre-treatment ALT level, stage of fibrosis, insulin resistance and genetic factors) may have an impact on antiviral treatment outcome. [3-5] One of the factors that may play an important role in re-infection of the liver and in the high rate of unsuccessful treatment and relapse in patients with CHC is active HCV replication in peripheral blood mononuclear cells (PBMC).[6] It is generally accepted that the presence of minus strand HCV RNA in hepatocytes and PBMCs may be an indicator of continued

proliferation of the virus, and eventual relapse in SVR patients.[6-7]

Based on current combination therapy, 65% of Iranian HCV infected patients achieve SVR, approximately 20% relapse after end of therapy (ETR), and the remaining 15% was non-responders (NR).[8] Due to the predominance of genotype 1a and 3a in Iran,[9] the prediction of NR, SVR and relapse would be useful to decide whether to continue therapy. Among immune factors, IFN-γ has elicited great interest in chronic viral infections which plays an important role as the first line of host defense to control viral infections.[10] This cytokine is abundantly produced especially by T helper (Th) and involved in the immune and inflammatory responses which strongly inhibit HCV replication and has direct antiviral activity against HCV, although it has not been approved by all the previous researches.[11-13] Recently, a distinct class of IFNs, the family of IFN-λ, has become the topic of intense investigation in the field of viral hepatitis. The family of IFN-λ consisted of IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) is known to possess potent antiviral activities in association with spontaneous and therapy-induced clearance of HCV.[14-15] We only examined IL-29, because of low and sometimes undetectable serum level of the other members of IFN-λ family.[16-17] In general, the first logical step to determine the role of endogenous IL-29 in triggering an effective antiviral response in chronic HCV is to measure the IL-29 level in the sera of chronically infected HCV patients and healthy individuals. However, there are conflicting results about the level of IL-29 measured in the sera of HCV patients and healthy individuals. Some reports indicated that HCV chronically infected patients have lower IL-29 level in the sera than acutely infected patients, spontaneously resolved infection patients and healthy individuals.[1, 17] On the contrary, it was reported that IL-29 serum level of HCV infected patient is elevated compared to those of healthy individuals.[18-19]

To understand these discrepancies and to identify the role of IFN-γ and IL-29 in outcome of therapy, we performed an analysis of IFN-γ and IL-29 sera levels in a group of HCV infected patients at ETR and during follow up. They were categorized according to their HCV treatment results status as relapsers, non-responders (NR) and SVRs. Also, the frequency of plus-/minus strand HCV RNA in PBMCs of these groups of patients was investigated.

Methods

Patients & Samples

A total of 49 HCV infected patients, as diagnosed by the presence of anti-HCV Abs and HCV-RNA in serum with no background disease who were followed up for at least eighteen months in the Digestive Disease Research Center (DDRC) were chosen. This study was approved by the hospital's ethics committee. Informed consent was obtained from all patients before inclusion in the study.

Patients with human immunodeficiency virus (HIV) infection, any possible cause of liver injury other than HCV and decompensate cirrhosis were excluded. Weeks 24 and 48 of therapy were chosen for their importance in distinguishing NR patients from patients who should continue to be monitored for 6 months after completion of the combination therapy in HCV infected patients with genotype 3a and 1a, respectively. Plasma HCV RNA level was measured using COBAS TaqMan HCV RNA assay (Roche COBAS Amplicor HCV Monitor v 2.0, Roche Diagnostics, Mannheim, Germany) before treatment, at ETR and 6 months after ETR. The status of HCV treatment results and the outcome of therapy can be assessed at various points throughout therapy. Those patients who failed to clear HCV RNA from their serum at ETR are NR patients. The SVR is defined as the absence of HCV RNA in serum by a sensitive polymerase chain reaction (PCR) at ETR and 6 months later,[20] and relapse patients attained undetectable HCV RNA at ETR but experience HCV RNA during follow up.[6] According to the definitions, 20 patients were SVRs, 15 patients were NRs and 14 patients experienced relapse.

Sample preparation

To prepare the PBMC, 10 mL of EDTA-treated blood was centrifuged through a ficoll density gradient. The cells were washed three times with a RPMI culture medium and adjusted to a final concentration of 2×10^6 cells. The PBMC pellet was aliquoted in RNA*later* (Qiagen) and stored at -80˚C as described previously.[21]

Extraction of nucleic acids and cDNA synthesis

Virus RNA was extracted from 2×10^6 PBMC using the High Pure Viral RNA Kit (Roche, Mannheim, Germany). The cDNAs were synthesized with specific primers (Table 1). RNA samples were incubated first for 5 min at 70˚C with 0.2 μM of primer and followed by incubation for 10 min at

Table 1: The sequence of specific primers and internal controls

Primers of first round of PCR	External Forward	5'-TCTCGTAGACCGTGCACCATGAGC-3'
	External Reverse	5'-AAGCCGCACGTAAGGGTATCG-3'
Primers of second round of PCR	Internal Forward	5'-GGTCAGATCGTTGGTGGAGTTTAC-3'
	Internal Reverse	5'-CGGGGAGACAGGAGCCA-3'
GAPDH Primers	Forward	5'-ACCTGACCTGCCGTCTAGAAA-3'
	Reverse	5'-CCTGCTTCACCACCTTCTTGAT-3'

37˚C with a reaction mixture containing 200 U M-MuLV RT (Fermentas, Lithuania), 1 mMdNTP, 20 U rRNAsin (Fermentas, Lithuania) and RT-buffer 1X (Fermentas, Lithuania). Samples were then incubated for 1 hour at 42˚C followed by heat inactivation for 5 min at 80˚C. Distilled water and normal PBMC were used as negative controls in each extraction. To amplify positive and negative strand HCV PCR, the reverse and forward primers were added to the RT reaction, respectively.

Positive and negative strand HCV PCR amplification in PBMCs

The obtained cDNA was amplified by nested-PCR using two pairs of primers recognizing the HCV-core region. One-seventh of the generated cDNA was amplified with the combination of primers (Table 1). Briefly, reaction mixture consisted of 1.5 mM $MgCl_2$, 0.2 mMdNTP, 1μM of each primer, 1 U of *Taq* DNA polymerase (Fermentas, Lithuania) and PCR-buffer 1X (Fermentas, Lithuania). The PCR conditions consisted of incubation at 95˚C for 2 min and 35 cycles of 25 seconds at 95˚C, 20 seconds at 58˚C, 50 seconds at 72˚C, and a final extension cycle of 72˚C for 7 minutes.

Second-Round PCR was conducted for each sample, using the first-round using PCR amplicon and specific primers. To increase the sensitivity of the PCR reaction, different parameters were optimized: The best concentration of $MgCl_2$ was found to be 1.5 mM and the best annealing temperature was 60˚C. Optimization program showed that 35 cycles and 0.25 μM concentration of primers are the best protocol for second amplification. The second round of PCR conditions standardized for amplification were an initial denaturation at 95˚C for 2 minutes and 30 seconds.; 35 cycles of denaturation (95˚C) for 25 seconds, annealing (60˚C) for 20 seconds, extension (72˚C) for 30 seconds followed by final extension at 72˚C for 7 minutes. Sensitivity of PCRs was determined using Probit Regression Analysis. The results indicated that the limit of detection of the test was 116 IU/ml of RNA.

To process and analyze the PCR products, the positive and negative controls were included among samples. Target gene amplification was confirmed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression using related primers (Table 1).

Detection of the interferon serum level

Both IL-29 and IFN-γ were measured in the serum of patients at ETR and six months later using the ELISA kit (eBioscience, San Diego, CA). Detection limits for IL-29 and IFN-γ were 8 pg/ml-1000 pg/ml and 4 pg/ml-500 pg/ml, respectively. Assays were performed according to the manufacturer's guidelines.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (version 15). Descriptive analysis including frequencies and cross tabs and parametric and non-parametric tests including chi-square, T-Test and logistic regression was carried out. A p-value less than 0.05 were considered to be statistically significant.

Results

Demographic & biochemical analysis

The clinical characteristics of the ETR patients are presented (Table 2). They consisted of 49 HCV infected patients who completed the entire treatment course. Among all treated patients, 38 (77.6%) were

Table 2. Clinical characteristics of 49 HCV-infected patients

CHARACTERISTIC		Groups of Patients			TOTAL
		NR	RELAPSE	SVR	
Mean age (range)		48.6 (32- 70) years	49.7 (31 - 63) years	36.2 (26 - 54) years	43.8 (26 - 70) years
Sex, n (%)	Males	10 (66.7%)	10 (71.4%)	18 (90%)	38 (77.6%)
	Females	5 (33.3%)	4 (28.6%)	2 (10%)	11 (22.4%)
HCV Genotype, n (%)	1a	10 (66.7%)	3 (21.4%)	16 (80%)	29 (59.2%)
	1b	3 (20%)	1 (7.1%)	2 (10%)	6 (12.2%)
	1a / 1b	2 (13.3%)	0	1 (5%)	3 (6.1%)
	3a	0	10 (71.4%)	1 (5%)	11 (22.4%)
Mean ALT, (IU/ml) (range)		38.8 (10-80)	57.5 (9-226)	27.1 (8-55)	39.9 (8 – 226)
Mean IFN-γ, (Pg/ml) (range)		21.7 (7-50)	107.2 (80-127)	152.7 (120-250)	99.6 (7 – 250)
Mean IL29, (Pg/ml) (range)		19.4 (11-30)	155.8 (135-185)	238.7 (200-272)	147.9 (11 – 272)
Mean HCV load, (Copies/ml) (range)		3.4×10^6 (2.1×10^4 – 9.1×10^6)	4.1×10^6 (1.7×10^4 – 14.9×10^6)	2.7×10^6 (2.9×10^3 – 16.9×10^6)	3.3×10^6 (2.9×10^3 – 1.6×10^7)
Patients with not documented HCV load		0	2	3	5 patients

* ALT, alanine aminotransferase

males and 11 (22.4%) were female. Patients were divided into two groups of NR (n = 15) and ETR (n = 34) at 24 and 48 weeks after therapy for genotype 3 and 1, respectively. Fourteen out of 34 ETR patients indicated evidence of virologic relapse and 20 patients achieved SVR after the completion of therapy in the follow up period. There was no significant correlation between final condition of treatment and sex or ALT level (chi-square, P = 0.173), but genotype of patients had an important role on SVR and the relapse. The patients with genotype 1a and 1b compared to 3a had a fifty (logistic regression, P = 0. 003) and twenty seven fold (logistic regression, P = 0. 035) greater chance to response to therapy, respectively. Opposed to genotype 1b, genotype 3a had a higher level of viral load and ALT at the baseline. Ten out of eleven (90%) patients with genotype 3a that were negative for HCV RNA at ETR showed relapse 6 months later. This result showed that the relapse outcome was significantly correlated with infection with the genotype 3 of HCV ($\chi^2 = 29.308$ $P < 0.0001$,).

Serum IFN- γ and IL-29 changes at ETR and six months later

The serum level of IFN-γ (*t*-test, $P < 0.0001$) and IL-29 (*t*-test, $P < 0.0001$) in ETR patients was significantly higher than NR patients. Among ETR patients, SVR patients had a higher level of IFN-γ and IL-29 (Figure 1), at ETR and 6 months later compared to those who relapsed.

The relapsed patients had an insignificant higher level of viral load and ALT at the baseline in compared to SVR. A significant (paired *t*-test, $P < 0.0001$) decline in the relapsed patients' IFN-γ and IL-29 serum levels were occurred during 6 months after ETR (Figure 2). In SVR patients IL-29 (paired *t*-test, $P < 0.0001$) and IIFN-γ (paired *t*-test, $P < 0.0001$) levels increased significantly during follow up.

Moreover, we found a correlation coefficient of 70% between IFN-γ and IL-29 levels in SVR and the relapsed patients at ETR and later (Figure 3). Although the baseline viral load and ALT level in NR patients was lower than the relapsed individuals and higher than SVR, there was no significant correlation.

Virologic analysis

The detectability of plus-/minus strand of HCV RNA was monitored in total RNA preparation of PBMC in 49 patients at ETR. Among patients who were positive for plus HCV RNA, detectability of minus strand RNA was evaluated, simultaneously (Table 3). Although HCV RNA minus strand was more common in NR than ETR, there was no significant correlation between the presence of plus (chi-square, P = 0.081) and/or minus strand (chi-square, P = 0.315) in ETR patients with outcome of therapy with the relapse or SVR. In other words, being positive for either plus-/minus strand at ETR, could not predict the outcome of therapy during follow up. Also there was no correlation between

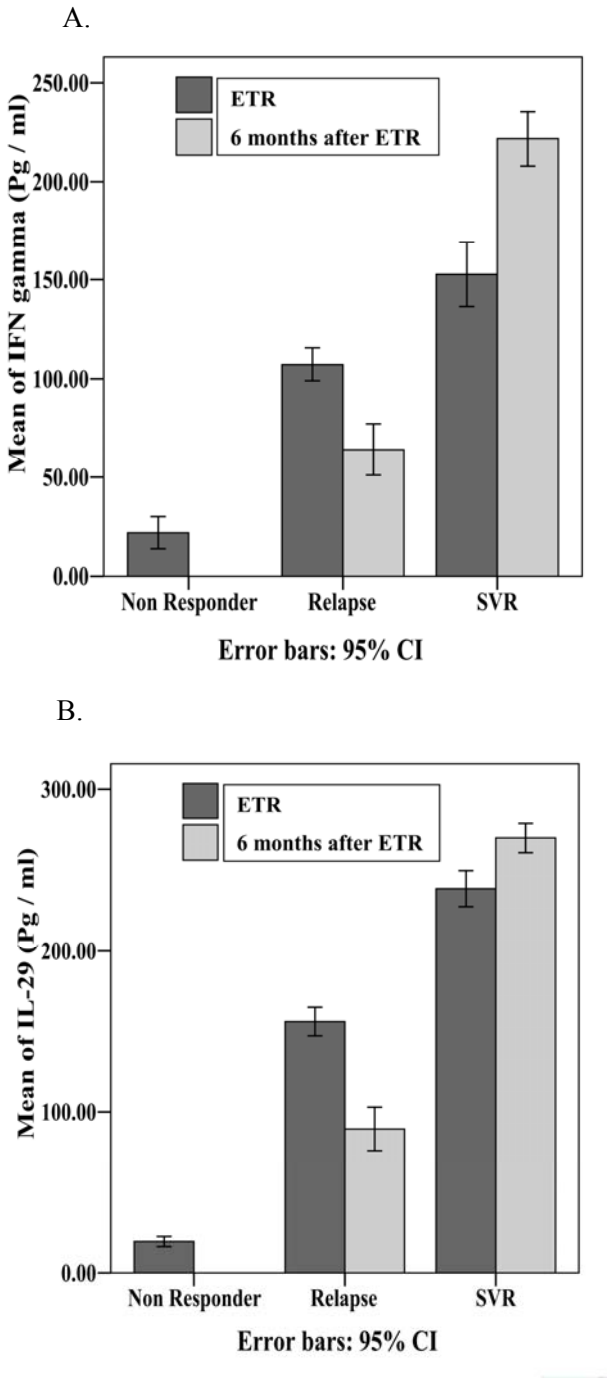

Figure 1. Changes in the IFN-γ (a) and IL-29 (b) level at ETR and 6 months later. SVR patients had a higher level of IFN-γ and IL-29 at ETR and during follow up compared to NR and the relapse patients. The level of IFN-γ and IL-29 was decreased during follow up in the relapse patients. A significant difference was observed between SVR and the relapse.

the baseline viral load of HCV and the presence of plus-/minus strand at ETR. Patients positive for minus strand of HCV RNA, had an insignificant higher viral load compared to others at ETR. The detectability of plus-/minus strand HCV RNA was monitored in PBMCs of 34 ETR patients 6 months after ETR. All of patients who had detectable minus strand in PBMCs 6 months after ETR were experienced relapse and all were infected with genotype 3a, too. In contrast, patients who achieved SVR were able to clear the minus strands (Table 3). The difference in presence of minus strand was statistically significant in mentioned groups during the follow up (chi-square, $P = 0.001$). The patients with undetectable either plus or minus HCV RNA had higher serum levels of IFN-γ and IL-29, but the correlation was not significant. The sex, genotype, age, viral load and ALT did not affect frequency of plus-/minus strands of HCV RNA at ETR.

Discussion

The pathogenesis of HCV has been postulated to be due to the interaction between viral factors and host immune response.[21] Relapse is one of the greatest concerns following successful treatment of HCV infection which clinical outcomes and pathology for optimal management are poorly understood.[3] Furthermore, the ability to distinguish between ETR responders with a probability of relapse is one of the important steps to implement interventions to reduce relapse rate.[3] To examine the role of endogenous IFN-γ and IL-29 in chronically infected HCV patients, we measured IFN-γ and IL-29 serum levels in 49 chronically infected HCV patients, 15 NR patients at ETR, 20 SVR individuals and 14 relapse patients at both ETR and 6 months later. Among the three different IFN-lambdas, IL-29 was chosen because the previous report has shown that in patients with chronic HCV, IL28A/B serum levels are very low comparing to IL-29 level.[16-17] Furthermore, we analyzed the correlation between the presences of plus-/minus strand HCV RNA in PBMCs of chronically HCV infected patients at ETR with outcome of therapy. We found that serum level of IL-29 was higher in patients who resolve HCV infection at ETR compared to NR patients. This finding is in agreement with some,[17] but not all recent studies.[22] The reason for this disagreement may be due to different times of measurement of IL29 as 0.25 μM concentration0.25 μM concentration et al.[22] investigated IL-29 serum level at baseline and after 12 weeks of therapy. The serum level of

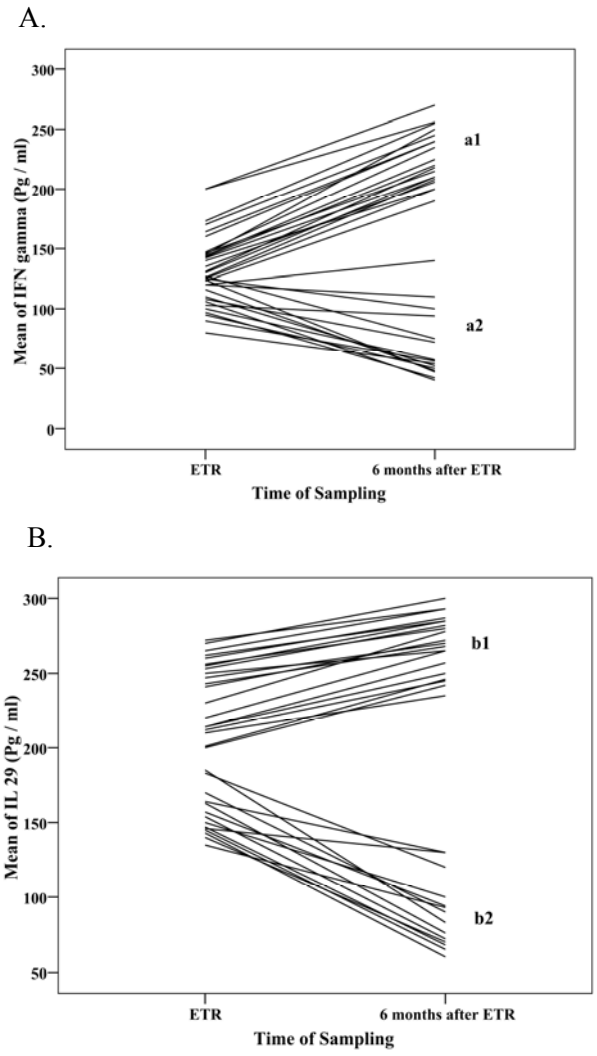

Figure 2. A) Changes in the serum level of IFN- γ at ETR and 6 months later in the SVR (a1) and relapse (a2). A significant difference was observed between the two groups (t-test, $P < 0.0001$).
B) Changes in the serum level of IL-29 at ETR and 6 months later in the SVR (b1) and relapse (b2). A significant difference was observed between the two groups (t-test, $P < 0.0001$).

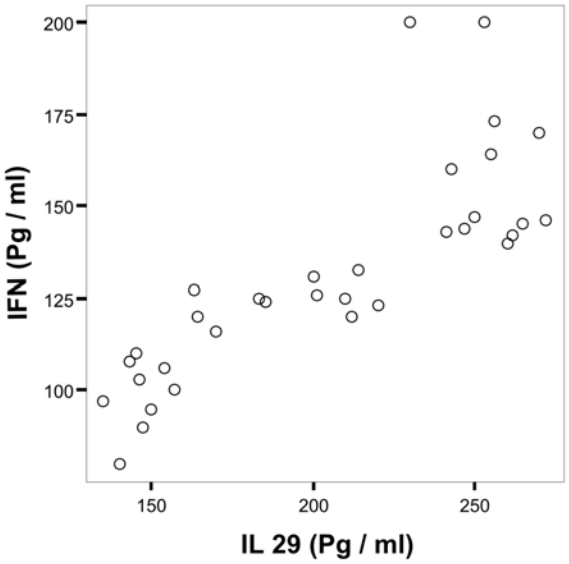

Figure 3. Correlation coefficient of 70% was observed between IFN- γ and IL-29 levels at ETR and during follow up in the relapse and SVR individuals.

IL-29 in SVR patients was higher than that of the relapsers, 6 months after ETR, suggesting that the level of IL-29 could be used to discriminate between the relapsed and SVR patients at ETR. It implies that IL-29 may play a role in antiviral immune responses against the HCV infection as supported by the strong correlation between the high level of IL-29 and spontaneous clearance of HCV.[16-17]

Langhans et al. reported that patients with acute hepatitis C showed an equal level of IL-29 between chronic hepatitis C and normal controls; and IL-29 serum level was higher in patients who spontaneously resolved hepatitis C than in those who became chronic suggesting that the high level of these cytokines predispose people to spontaneous resolution of HCV infection.[17] On the other hand, in the report of Torres et al., IL-29 serum level was not significantly different between SVR and NR patients at 12 weeks of therapy suggesting that IL-29 does not play an important role in viral elimination during treatment, at least not during the first 12 weeks of therapy.[22] It is not clear whether the IL-29 serum level would experience significant changes beyond 12 weeks. Also, it showed that IL-29 in patients who achieved SVR was not higher at week 12 of treatment than at baseline level.22 We found that the IFN-γ level was significantly higher in SVR than the relapsed and NR at ETR and later. Zhang et al. and Perrella et al. showed that serum level of IFN-γ in SVR is higher than NR.[23-24] On the other hand, a previous study by Wan and colleagues and Fathy et al. stated that there was no higher level of IFN-γ in SVR compared to NR.[13,25] This different result might be attributed to the differences in the sample size which was relatively small (26 patients) in Fathy et al. report, [25] the extent of histological liver damage, and the time of the assessment of IFN-γ serum level which was earlier (12 weeks) than the usual proper duration. Another new finding of this study was that, in addition to the serum level of IL-29, the level of IFN-γ at ETR probably could predict

Table 3. Virologic analysis of plus-/minus strand of HCV RNA in PBMCs of patients

Time	at the end of treatment			at 6 months after end of treatment		
	NR	ETR	Total	Relapse	SVR	Total
	n=15	n=34	n=49	n=14	n=20	n=34
Plus Strand	11 (73.3%)	16 (47%)	27 (55.1%)	11 (78.5%)	4 (20%)	15 (44.1%)
Minus Strand	8 (72.7%)	4 (25%)	12 (44.4%)	6 (54.5%)	0	6 (40%)

relapse occurence from SVR at ETR as the serum level of IFN-γ and IL-29 in the relapse group was significantly lower than SVR patients 6 months after ETR. From a practical point of view, it should be helpful to discriminate patients treated with a combination therapy of PEG-IFNα and ribavirin at ETR with expected relapse outcome six months after ETR from SVR to make new strategy of treatment. In support to this finding, CC IL-28B polymorphism has been reported to have a strong correlation with SVR and instead, CC genotype carriers had higher level of IL-29 than TT genotype carriers.[17]

Thus the present study opens the interesting possibility of improvement of discrimination of relapse to combination therapy by using easily assessed immune response biomarkers such as IFN-γ and IL-29 to make the decision to continue or resume therapy. We also assessed plus-/minus strand HCV RNA with the SVR, NR and relapse outcome. Since HCV can replicate in PBMCS,[26] the goal of anti-viral therapy is to decrease replication of HCV RNA strands; so monitoring of PBMCs with the reservoir role for replication of HCV is important.[26-28] Our results did not show a significant correlation between the presence of plus-/minus strand HCV RNA in PBMCs and relapse or SVR to treatment in HCV-infected patients after termination of antiviral therapy. Also the presence or absence of minus strand of HCV RNA in patients at ETR was not correlated with the outcome of therapy. This result is compatible with the report of Gil et al. (1993) that the patients experienced relapse despite the absence of negative HCV strand in the PBMCs and liver biopsy.[29] In the study of García-Bengoechea et al. (1999) detectable plus strand of HCV RNA in PBMCs during the follow up was not related to the relapse outcome.[30] It seems that IFN therapy has an inhibitory effect on HCV replication in PBMCs, but does not achieve elimination of HCV infection.[31] In concordance with our result, there are some reports that showed that HCV RNA was detectable in patients with SVR.[31-33] Despite the different results, the conclusion is the same: HCV RNA determination in PBMCs is not a predictor of SVR and the relapse outcome; thus, SVR patients presenting detectable HCV RNA and patients with undetectable HCV RNA in PBMCs can experienced relapse. On the other hand, in the study of Amini et al. (2012), it was shown that the presence of plus strand HCV RNA in PBMCs was associated with viral relapse.[6] In another study, Xu and et al (2005) reported that 8 of 9 (88.9%) patients with HCV RNA in PBMCs showed evidence of viral relapse during the follow up period.[26] El-Awady et al. indicated that patients, who had HCV RNA in PBMCs at ETR, are more susceptible to switch to relapse in one year after ETR. They conclude that PBMCs serve as a reservoir for HCV replication.[34]

Despite some reports that virological relapse is more common among genotype 1 than genotype 3,[35] we found that genotype 3 compared to genotype 1 had a response predictor role for relapse outcome. It is possible that the small number of patients studied in this research explains the difference. We did not find any association between sex, genotype, age, baseline viral load and ALT with frequency of HCV RNA strands.

Defining parameters that predict relapse to IFN-α therapy in patients with chronic hepatitis C has been intensively sought during the last few years. To our knowledge, this study is the first to address the IL-29 and IFN-γ serum levels and viral genomic HCV present in the PBMCs of three patients groups, those who had SVR not responding to therapy and those who presented relapse.

In conclusion, there was no significant difference between presence of plus-/minus strands of HCV RNA in SVR, relapse or non-responder groups at ETR. Besides, we showed that the serum levels of IFN-γ and IL-29 at ETR are probably useful to discriminate patients with relapse from those with SVR at ETR. In other words, IFN-γ and IL-29 can be regarded as an attempt by the immune system to

block HCV replication and to eradicate the virus as well as a predictive outcome factor for the responsiveness of HCV patients to combination therapy at ETR. Because the small sample size used in this study, more research is needed to support the predictive role of IFN-γ and IL-29 on the outcome of HCV infected patients. However, low levels of IFN-γ and IL-29 in sera of those who were negative for HCV RNA at ETR can be considered as a biomarker to predict the relapsed outcome after ETR and whether to continue therapy for a longer duration for the relapsed individuals to achieve the optimal outcome.

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Conflict of interest

We have no conflict of interest to declare.

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