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The Role of Polymorphisms Near *IFNL3* Gene as Predictors of Residual HCV RNA in Buffy Coat after Successful Antiviral Therapy

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Abstract

Background and Aims: The presence of the hepatitis C virus (HCV) in cells of extrahepatic organs like peripheral blood mononuclear cells (PBMCs) have important implications for transmission, disease progression, and effective treatment of HCV-infected patients. The impact of host genetics such as polymorphisms near Interferon lambda 3 (*IFNL3*) on clearance of HCV RNA from buffy coat (BC) following successful clearance of HCV from plasma using Pegylated-IFN (PegIFN) and Ribavirin (RBV) treatment was evaluated in our study.

Methods: For detection of residual HCV RNA in BC samples, blood samples of 69 patients with sustained virologic response (SVR) after treatment with PegIFN and RBV were evaluated. Polymorphisms near *IFNL3* gene including rs12979860 and rs8099917 were assessed using PCR-RFLP method.

Results: The most prevalent rs12979860 and rs8099917 genotypes were CT (49.3%) and TT (62.3%), respectively. Nine (13.04%, 95%CI: 7.01% - 22.96%) patients had HCV RNA in their BC samples. The favorable genotypes of the 2 polymorphisms (rs12979860 CC and rs8099917 TT) were more frequently observed in patients with undetectable HCV RNA in their BC samples than those with HCV RNA in their BC samples (rs12979860 CC, 45% vs. 22.2%, P = 0.016 and rs8099917 TT, 66.7% vs. 33.3%, P = 0.01).

Conclusions: The polymorphisms of *IFNL3* could play a crucial role not only in spontaneous clearance of HCV and SVR rate after PegIFN and RBV therapy, but also in the clearance of HCV from BC after PegIFN and RBV therapy.

Keywords: Polymorphisms, *IFNL3*, HCV, PBMC, rs12979860, rs8099917

1. Introduction

Hepatitis C virus (HCV) is a single-stranded hepatotropic RNA virus, which causes chronic hepatitis in more than 70% of infected individuals (1). It has been estimated that 2.2% of the world population are suffering from chronic HCV infection. The incidence rate of HCV infection can be found as the highest in the eastern mediterranean region (EMRO) and Africa (2, 3). Seroprevalence of HCV in Iran varies from 0.2% to 1.5% and HCV is the most important reason for chronic liver disease in hemophilia, thalassemia, patients with renal failure, and those on hemodialysis (4). The patients with chronic hepatitis C infection should be treated with antiviral agents to prevent the progression of liver diseases (5). The available treatment regimens contain immunomodulatory agents such as interferon (IFN) and Pegylated-IFN (PegIFN) and/or direct-acting antiviral agents (DAAs) such as NS3 protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors (6). The antiviral therapy could clear HCV from patient's plasma while it is not obvious whether it can result in eradication of the virus from the patient's liver and/or other extrahepatic reservoirs (7-9).

The natural history and outcome of HCV infection can be determined by various parameters of virus and host (10). The same is true for traditional treatment with IFN-based regimens (11, 12). Among HCV parameters, HCV genotype, HCV RNA level, and variations in HCV genome are able to change response to PegIFN and ribavirin (RBV) combination therapy (13, 14). Host factors such as gender, age, ethnicity, and genetic polymorphisms have remarkable effects on the outcome of HCV infection as well (11, 13, 15, 16). Since 2009, many studies showed the important role of polymorphisms near Interferon lambda 3 (*IFNL3*) in spontaneous and IFN-based treatment-induced clearance of HCV infection (14, 17, 18). Many studies investigated the

impact of host genetics on clearance of HCV RNA from plasma but no study investigated the impact of host genetic parameters such as polymorphisms near *IFNL3* on the clearance of HCV RNA from peripheral blood mononuclear cells (PBMCs) or buffy coat (BC) following successful clearance of HCV from plasma using treatments.

2. Objectives

This study aimed to investigate the prevalence of residual HCV RNA in BC samples following successful clearance of HCV RNA from plasma in patients treated with PegIFN and RBV. Moreover, the impact of polymorphisms near *IFNL3* on the clearance of HCV from BC was evaluated.

3. Methods

3.1. Study Population

Retrospectively, 69 patients with cleared HCV infection from plasma following treatment with PegIFN and RBV (within 2012 to 2013) were studied cross-sectionally. Censusly and from all patients referred to the referral center of Tehran hepatitis clinic, we assumed the below inclusion criteria: 1) patients with chronic HCV infection (detectable HCVAb and HCV RNA for more than 6 months) prior hepatitis C treatment, 2) chronic hepatitis C patients who were treated with PegIFN and RBV, and 3) patients with sustained virologic response (SVR). The exclusion criteria were as follows: 1) HCV/HIV coinfection, 2) HCV/HBV coinfection, 3) HCC, and 4) liver transplantation.

Patients were treated with PegIFN-α2a or -α2b and RBV for 6 to 18 months based on the HCV genotype and on-treatment response. The study population was selected consecutively from patients treated at the Tehran hepatitis clinic (Tehran, Iran) considering the above-mentioned inclusion and exclusion criteria. Undetectable HCV RNA, 6 months after treatment cessation was considered as SVR, which indicated treatment success. The ethics committee of the Baqiyatallah research center for gastroenterology and liver diseases approved the study design and ethical approaches. All study participants provided informed consent explaining the aims of our study. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki declaration of 1975, as revised in 2008.

3.2. Laboratory Assessments

For detection of residual HCV RNA in BC samples, blood sample with ethylenediaminetetraacetic acid anticoagulant was collected from patients, 6 - 36 month after achieving SVR. The BC was separated after 10 minutes of centrifugation at 3000 RPM. Total nucleic acid was extracted from BC using QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The extracted nucleic acid was subjected to cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Fermentas of Thermo Fisher Scientific, Waltham, MA, United States). Hepatitis C virus 5'-untranslated region (5'-UTR) was detected in the product of cDNA synthesis using QIAGEN® OneStep RT-PCR Kit (Qiagen, Hilden, Germany) and the following primer set: 3'-AGCGTCTAGCCATGGCGT-5' and 3'-CAAGCACCCTATCAGGCAGT-5'. The amplification of HCV 5'-UTR resulted in a 234 base pair (bp) DNA fragment, which was detected on a 3% agarose gel.

In this study, rs12979860 and rs8099917 polymorphisms were assessed as the most common polymorphisms near *IFNL3* gene. The detailed protocol of the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for genotyping of rs12979860 and rs8099917 polymorphisms was previously described (19).

3.3. Statistical Analysis

Categorical variables were expressed as frequencies and percentages, and continuous variables were expressed as the median (interquartile range). The Fisher's exact test was used to analyze the categorical variables and the Mann-Whitney U test was utilized to analyze continuous variables. The Hardy-Weinberg Equilibrium (HWE) was assessed for the rs12979860 and rs8099917 SNPs, and the linkage disequilibrium (LD) between these SNPs was calculated. P values less than 0.05 ($P < 0.05$) were statistically significant. Statistical analyses were performed using SPSS version 20.0 (IBM SPSS, Chicago, IL, USA).

4. Results

Patients' baseline characteristics and on-treatment response were included in Table 1. Most of the studied patients were male and young with a median age of 24. Most of the patients had HCV genotype 1 infection prior to antiviral therapy. The most prevalent rs12979860 and rs8099917 genotypes were CT (49.3%) and TT (62.3%), respectively (Table 1). The distributions of both rs12979860 and rs8099917 genotypes were in HWE ($P = 0.37$ and $P = 0.21$, respectively) and the 2 SNPs were in moderate LD ($D' = 1.0$, $r^2 = 0.49$). During treatment, 95.6% of patients achieved complete early virologic response (cEVR).

Among study population, 9 (13.04%, 95%CI: 7.01% - 22.96%) patients had HCV RNA in their BC samples. To assess the factors that determined the persistence of HCV

Table 1. Baseline Characteristics and on-Treatment Response of the Study Population in Relation to Detection of HCV RNA in Buffy Coat

	All (n = 69)	HCV RNA in BC		P Value
		Not detected (n = 60)	Detected (n = 9)	
Sex[a]				0.582[b]
Male	62 (89.9)	53 (88.3)	9 (100.0)	
Female	7 (10.1)	7 (11.7)	0	
Age, y,[c]	24.0 (12.0)	24.0 (11.5)	24.0 (8.0)	0.532[d]
Serum ALT,[e] **IU/L**[c]	42.5 (42.0)	42.0 (45.0)	46.0 (12.0)	0.871[d]
Serum AST,[e] **IU/L**[c]	31.0 (20.0)	32.0 (19.0)	31.0 (18.0)	0.885[d]
HCV RNA, Log IU/mL[c]	5.9 (0.7)	5.9 (0.7)	5.8 (0.7)	0.428[d]
HCV genotype[a]				0.452[b]
1	47 (68.1)	42 (70.0)	5 (55.6)	
2/3	22 (31.9)	18 (30.0)	4 (44.4)	
rs12979860[a]				0.016[f]
CC	29 (42.0)	27 (45.0)	2 (22.2)	
CT	34 (49.3)	30 (50.0)	4 (44.4)	
TT	6 (8.7)	3 (5.0)	3 (33.3)	
rs8099917[a]				0.010[f]
TT	43 (62.3)	40 (66.7)	3 (33.3)	
GT	25 (36.2)	20 (33.3)	5 (55.6)	
GG	1 (1.4)	0 (0)	1 (11.1)	
History of antiviral therapy[a]				0.891[f]
Naive	56 (82.1)	49 (81.7)	7 (77.8)	
Relapse	8 (11.6)	7 (11.7)	1 (11.1)	
Non-response	5 (7.2)	4 (6.7)	1 (11.1)	
cEVR[a,e]				> 0.999[b]
Yes	65 (95.6)	56 (94.9)	9 (100)	
No	3 (4.4)	3 (5.1)	0	

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BC, buffy coat; cEVR, complete early virologic response; IQR, inter quartile range; n, number.
[a]Values are expressed as No. (%).
[b]Fisher-exact test.
[c]Values are expressed as median (IQR).
[d]Mann-Whitney U test,
[e]The data were missed in less than 10% of patients.
[f]Chi-Square.

RNA in BCs, we compared the patients' baseline characteristics and on-treatment response (cEVR) between patients with residual HCV RNA in their BC samples and those with undetectable HCV RNA in their BC samples. In terms of the gender, age, HCV genotype, history of antiviral therapy, and levels of serum transaminases and HCV RNA, there was no difference between the 2 groups ($P \geq 0.05$; Table 1). Moreover, achievement of cEVR did not influence the rate of detection of HCV RNA in BC samples of patients with SVR to PegIFN and RBV treatment. However, the favorable genotypes of the 2 polymorphisms (rs12979860 CC and rs8099917 TT) near *IFNL3* were more frequently observed in patients with undetectable HCV RNA in their BC samples than those with HCV RNA in their BC samples (rs12979860 CC, 45% vs. 22.2%, P = 0.016 and rs8099917 TT, 66.7% vs. 33.3%, P = 0.01) (Table 1). Moreover, patients with HCV RNA in their BC samples were more frequently harbored rs12979860 TT and rs8099917 GG genotypes than patients without HCV RNA in their BC samples (rs12979860 TT, 33.3% vs. 5%, P = 0.016 and rs8099917 GG, 11.1% vs. 0%, P = 0.01) (Table 1).

5. Discussion

Replication of HCV in PBMC in spite of clearance of the virus from plasma has been described as occult HCV infection (OCI) in recent years (8, 20). Despite the fact that hepatocytes are considered as the main target of HCV, experimental and clinical evidences strongly point to the presence of virus in cells of extrahepatic organs for invading and replication, particularly the immune system (21). In fact, extrahepatic reservoirs have important implications for transmission, disease progression, and effective treatment of HCV-infected patients. Clearance of HCV following antiviral treatment does not mean eradication of the virus from whole reservoirs (22). The presence of HCV RNA in PBMCs may lead to HCV reactivation in patients with SVR under special circumstances, such as immunosuppression (23).

In this study, although all patients had undetectable HCV RNA in their sera after achieving SVR with PegIFN and RBV, HCV RNA was still detected in isolated BCs of 13% of these patients. In contrast to our study, Inglot et al. (24) found 6.1% of patients with SVR to PegIFN and RBV had HCV RNA negative strand in their PBMC samples 24 weeks after termination of treatment. In another study, Fujiwara et al. (25) revealed that residual HCV RNA was not detected in plasma or PBMCs of any spontaneous or treatment-recovered subjects suggesting that the classic pattern of recovery from HCV infection is generally equivalent to viral eradication. Gallegos-Orozco et al. (23) included 25 patients with SVR to PegIFN and RBV treatment 6 - 56 months (mean, 22 months) after the end of treatment and looked for HCV RNA in their PBMC samples following cell culture. They observed the persistence of viral RNA in the PBMCs of 5 (20%) patients. Mohamad et al. (26) described that 26% of the SVR patients had a detectable level of HCV RNA in PBMC 6 month after completion of treatment. The current and all the mentioned studies, except the study done by Fujiwara et al. (25), found HCV RNA in the blood cells of the patients treated with IFN-based treatments. The difference in the reported rate of detection by these studies was mainly justified by the following points; different time points for detection of HCV RNA in PBMC after achieving SVR, various methods for detection of HCV RNA in the PBMC samples and different host and viral parameters of patients included. The question remaining is why the virus could be detected later in PBMC while cleared from the serum. One probable explanation is that HCV has developed a number of evasion mechanisms, infection of PBMCs being one of those where the virus can avoid the immune defense system, while hepatocytes remain the actual target. Previous works demonstrated that patients with seronegative HCV have an HCV-specific cellular immune response with a probable immune surveillance function, suggesting that host immune response is able to control but not to eliminate, HCV replication in these cases (27). It may also be that the virus developed some new quasi-species in PBMC that showed delayed response to the antiviral therapy but we could not confirm that speculation now.

The distribution of polymorphisms near *IFNL3* in the current study was the same as the previous studies (28, 29) in Iranian patients with HCV infection. Based on our observations, polymorphisms near *IFNL3* were associated with persistence of HCV RNA in BC samples of patients with SVR. The rs12979860 TT and rs8099917 GG, which classically are associated with treatment failure, were more frequently observed in patients with detectable HCV RNA in their BC samples. We hypothesized that polymorphisms near *IFNL3* could play a very crucial role not only in spontaneous clearance of HCV and SVR rate after PegIFN and RBV therapy, but also in the clearance of HCV from BC after PegIFN and RBV therapy. In the study done by Angulo et al. (30), 79% of hepatitis C viremic patients had HCV RNA in their PBMC samples. In this study, patients with rs8099917 TG/GG were more frequently observed to have HCV RNA in their PBMC samples at the point of viremia. The mechanisms in which *IFNL3* and host genetic variations causes such observation is not clear yet however discovery of *IFNL4* gene near *IFNL3* harboring rs12979860 and rs368234815 resulted in elucidation of different profiles of interferon stimulated genes expression, associated with treatment response to IFN-based regimens, by polymorphisms near or in *IFNL3*/*IFNL4* genes (29, 31-33).

Unexpectedly we could not find any association of baseline viral load and other clinical parameters with final clearance of virus, while some researchers have shown an association of OCI with high cholesterol and triglyceride levels (34).

There were few limitations in this study including: 1. The small sample size for the included patients with SVR, 2. We did not evaluate the HCV genotypes of the isolates from BC samples, and 3. The time span of blood sampling from the time point of achievement of SVR was not documented. The treatment of HCV has been revolutionized in the recent years and PegIFN and RBV will be not used as standard of care for treatment of patients with HCV infection. The new treatments are consisted of a combination of 2 or 3 direct-acting antiviral agents (DAAs), which can result in more than 90% SVR rate in patients with different condition of HCV infection (35). We believe that same study can be conducted in patients treated with DAAs. Moreover, it is crucial to evaluate the rate of HCV relapse in patients with SVR who has detectable HCV RNA in their PBMC samples especially under the circumstance of immunosuppression. In addition, it should be investigated if these patients have

a higher chance of cirrhosis and HCC in long-term follow-up.

5.1. Conclusions

Hepatitis C virus can be found in other reservoirs such as BC in a proportion of patients after successful treatment with PegIFN and RBV. Moreover, it seems *IFNL3* polymorphisms can determine the persistence of HCV RNA in BC samples in patients after successful treatment course of PegIFN and RBV. The reactivation of HCV infection in patients harbor unfavorable *IFNL3* genotypes following successful clearance of HCV viremia could be evaluated.

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