



Molecular Study of Occult Hepatitis C Infection among Iranian Hemophilia Patients Treated with Direct-acting Antiviral Agents

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ABSTRACT

Introduction: A new pathological form of HCV named as occult HCV infection (OCI) has been recently characterized by the presence of HCV RNA in liver biopsy and/or peripheral blood mononuclear cell specimens (PBMCs) and the absence of detectable circulating HCV RNA in plasma samples. In this study, we investigated the presence of HCV RNA in PBMCs and plasma samples of 100 hemophilia patients with negative serum HCV RNA. **Methods:** One hundred hemophilia participants receiving IFN-free direct-acting antivirals (DAAs) regimens as a treatment of HCV infection participated in this study. PBMCs were separated with Ficoll before RNA extraction. The HCV genotypes of the positive specimens were also analyzed by RT-PCR assay. Finally, data analysis was performed by SPSS software. **Results:** Our data revealed that out of 100 hemophilia patients, three (3%, 95% CI: 0.006-0.085) were positive for OCI, showing a significant association between OCI and genotype3/drug regimens ($p = 0.0203$). There was no significant increase in ALT and AST levels in patients with OCI. Moreover, a genotype difference was observed between plasma and PBMCs samples of 1% (1/100) of patients. **Conclusion:** Generally, HCV genotyping in PBMCs along with plasma subtyping before beginning the therapy is vital due to the possibility of OCI detection.

INTRODUCTION

Hemophilia is a hereditary blood disorder caused by missing or defective coagulating factors VIII and IX with a continual need for injectable blood derivatives associated with an increased incidence of blood-borne disease transmission [1-4]. Hepatitis C virus (HCV) from the Flaviviridae family is one of the risk factors in hemophilia patients requiring blood transfusion [5, 6]. Inhibiting the key viral replication targets such as NS3-4A protease, NS5A protein, and NS5B polymerase has led to a new HCV treatment strategy entitled as direct-acting antivirals (DAAs) [7, 8]. The primary goal of treatment is to eradicate HCV virus from the serum and to achieve a sustained virological response (SVR) defined as a viremia 12-24 weeks after completion of antiviral therapy for HCV infection [9, 10]. The introduction of the pan-genotypic direct-acting antiviral drugs known as IFN-free treatment was a revolution in HCV treatment since 2013. In contrast to previous regimens such as injectable interferon, DAAs are once-daily, all-oral, more effective, and less toxic [11, 12]. There are four classes of DAAs such as NS3/NS4A serine protease inhibitors, NS5A

inhibitors, NS5B polymerase inhibitors (both non-nucleoside agents and nucleoside/nucleotide analogs), and cyclophilin inhibitors [13]. The recent studies showed that HCV is the only human tumor virus that can be completely eradicated from infected cells by novel interferon-free DAAs regimens having achieved the SVR of over 90% [14]. HCV mainly replicates in hepatocytes, but it can reproduce in peripheral blood mononuclear cells (PBMCs), as well [15]. A new pathological form of HCV infection was reported by Pham *et al.* [16] for the first time described as occult HCV infection (OCI). Occult HCV infection is characterized by the presence of HCV RNA in liver biopsy and/or PBMCs specimens, and the absence of detectable circulating HCV RNA in plasma samples [17, 18]. Also, the activation of OCI may be the origin of late relapse [19]. Although the detection of HCV RNA in liver cells was considered as the gold standard, Castillo *et al.* (2010) suggested that testing for HCV RNA in PBMCs allows the diagnosis of occult HCV infection without the need for performing liver biopsy [20].

OCI was reported among various groups. For instance, the incidence of OCI was determined about 10.1% [21] and 57% [16] in cryptogenic liver diseases, 10.1% [22] and 18.2% [23] in hemodialysis patients, 1.9% [2] and 20% [24] in lymphoproliferative disorders, 0.25% [25] in kidney transplant patients, 8.9% [26] in liver transplant with cryptogenic cirrhosis, 39.0% [27] in glomerular nephropathies, 28.0% [28] in HBV-infected patients, 10.2% [29] in HIV-infected patients, and 3.3% [30] in general population.

Despite the significant progress achieved in HCV treatment, chronic HCV infection is still a major health problem in high-risk groups such as hemophilia patients. In the era of DAAs therapy for HCV infection, more than 90% of treatment was successful, and patients achieved a sustained virological response (SVR). In this study, we aimed to investigate the prevalence of occult hepatitis C in Iranian hemophilia patients treated with DAAs regimens by detection of viral RNA in their peripheral blood mononuclear cells to monitor treatment and to evaluate the need for this test for the assessment of a real cure in the patients.

MATERIAL AND METHODS

Patient selection. This descriptive cross-sectional study was conducted from a cohort of 100 hemophilia participants receiving IFN-free direct-acting antivirals (DAAs) regimens as a treatment of HCV infection from Iranian Comprehensive Hemophilia Care Center (ICHCC), Tehran, Iran. All the participants treated with DAAs achieved SVR (undetected serum HCV RNA 12 weeks after the end of treatment). One hundred men were included in this research. Before treatment, all patients were subjected to full history-taking and clinical examination.

Ethics approval. The Ethics Committee of ICHCC approved the protocol of the study (code: 135). We conducted all procedures according to the ethical principles of the Declaration of Helsinki, as revised in 2013. Written informed consent was received from all participants before inclusion in the study.

Serological tests using ELISA. Five ml of whole blood were collected from each participant into plain tubes. The plasma samples were analyzed for the presence of HBsAg, HIV, and HCV antibodies according to the manufacturer's instructions using enzyme-linked immunoassay (ELISA) kits (Dia. Pro, Diagnostic BioProbes Srl, Milano, Italy) in single wells. There was no evidence for cross-reactivity between the tests.

Preparation of PBMCs and plasma samples. Five ml of the peripheral blood sample was collected from each individual for PBMCs and plasma preparation into the tube containing ethylene diamine tetra-acetic acid (EDTA) as an anticoagulant. The plasma and PBMCs samples were

separated using the standard method of Ficoll Hypaque density gradient centrifugation (LympholyteHTM; Cedarlane and Hornby; Canada) [31] including phosphate-buffered saline (PBS) washing steps prior to cryopreservation in 10% dimethyl sulphoxide (DMSO, Sigma, Germany). The cells were washed three times with PBS to remove any contamination in the plasma and then kept at -80°C for future work.

RNA extraction from PBMCs and plasma samples. Total viral RNA was extracted from PBMCs and plasma samples using blood and biological fluids isolation kit (NucleoSpin® blood kit, MN, Germany) following the manufacturer's instructions. The resulting RNA pellets were dissolved in RNase-free water.

Molecular assay using the one-step RT-PCR method. One-step RT-PCR was performed using quantification of the 5' non-coding region (5' UTR) of the genomic HCV RNA designed by NCBI-Primer BLAST database as described in table 1.

The validity of sequences was investigated by SnapGene® 3.2.1 software. One-step RT-PCR amplifications were performed using 10 µl of RNA as a template in a total amount of 20 µl PCR Master Mix (Favorgen, Taiwan), and 10 pMol of each primer in the presence of additional standard PCR reagents provided by the supplier. The RT-PCR was performed at 55°C for 40 min, and amplified as follows: Initial denaturation step for 2 min at 94°C followed by 45 cycles of 94°C for 30 s, primer annealing at 57°C for 20 s, and extension at 72°C for 30 s, final extension was performed at 72°C for 1 min. A negative control (no RNA) was included in each PCR to assure the specificity of the results.

HCV genotyping. The HCV positive samples before and after DAAs treatment were selected for HCV genotyping according to the type-specific PCR method described by others [32]. At the first step, the HCV RNA genomes were reverse transcribed by Omniscript RT Kit (Qiagen, Germany). Then, two rounds of type-specific PCR were performed on cDNA samples. Ten microliters of the second-round PCR products were loaded on 2% agarose gel electrophoresis. HCV genotypes were determined by visualizing the HCV genotype-specific PCR band in agarose gel [32].

Statistical analysis. We performed all statistics by using SPSS 22.0 (SPSS Inc, Chicago, IL, USA). The qualitative variable was presented as a percentage. Statistical significance for comparison between groups was analyzed by the student's *t*-test, and differences in categorical variables were analyzed by Fisher's exact test. The 95% confidence interval was calculated based on the binomial distribution for occult HCV infection. Epidemiological and clinical data of the patients, including age, gender, levels of AST, ALT, and previous history of blood transfusion, were collected. The *p*-value below 0.05 was regarded as statistically significant.

Table 1. One-step PCR oligonucleotide primers to detect HCV

Primer Name	Sequence (5' to 3')	Product size (bp)	HCV genomic regions
HCV-Forward	GAAAGCGTCTAGCCATGGCGTTAGT	250	5'UTR
HCV-Reverse	CTCGCAAGCACCTATCAGG		

RESULTS

Patients' characteristics. This study involved 100 patients with the mean age (\pm SD) of 37 (\pm 10.50), ranged 23 to 64, treated with DAAs achieved SVR (undetected serum HCV RNA 12 weeks after the end of treatment). The full history of patients and clinical examination are summarized in table 2. Then, plasma samples were analyzed for the presence of HBsAg, HIV, and HCV antibodies using commercially available ELISA kits. All patients included in this study were negative for HBsAg, HIV and positive for HCV antibody.

Molecular studies. The blood samples were collected before and 12 weeks after receiving DAAs to compare the results with the following regimens: daclatasvir/sofosbuvir \pm ribavirin (DCV/SOF \pm RBV) in 28 patients and ledipasvir/sofosbuvir \pm ribavirin (LDV/SOF \pm RBV) in 72 patients. HCV RNAs were extracted from PBMCs samples and analyzed by RT-PCR. Three out of 100 patients (3%, 95%

CI: 0.006-0.085) who achieved SVR were positive for HCV RNA in PBMCs, consequently, positive for OCI. The liver enzymes of OCI patients were measured by using a commercial kit (Sigma, Germany). The mean levels of AST and ALT were 30.94 and 28.77 IU/L, respectively indicating normal activity of AST and ALT among patients with OCI. All three OCI patients had the HCV genotype 3a before their treatment. They had a history of blood transfusion, plasma injection, and drug regimen of DCV/SOF + RBV. The statistical analysis showed a significant association between OCI and genotype3/drug regimens (p -value=0.0203); however, there was no significant relationship between OCI and history of blood transfusion/plasma injection or even severity of hemophilia (Table 3).

In addition, one of the patients had different HCV genotypes in the plasma sample before the treatment (3a) and PBMCs after the treatment (1a). The detailed information on the patients with OCI is shown in Table 4.

Table 2. Demographic parameters and laboratory tests of the patients

Category	Data
No. of patients	100
Age, year (mean \pm SD)	37 \pm 10.50
Sex	Male
Severity of hemophilia	Severe 81 Mild and moderate 19
History of Blood Transfusion	Yes 85 No 15
History of Plasma injection	Yes 68 No 32
Liver-enzyme levels, mean \pm SD, IU/L:	Aspartate aminotransferase (AST) 30.94 \pm 19.91 Alanine aminotransferase (ALT) 28.77 \pm 22.45 Total bilirubin, mg/dL 1.53 \pm 1.08 Direct bilirubin, mg/dL 0.32 \pm 0.17
Infections	HIV Negative HBV (HBsAg) Negative HCV Antibody Positive

Table 3. Distribution of Occult HCV infection among Iranian hemophilia patients treated by direct-acting agents

		OCI positive (n)	OCI negative (n)	p -value	CI (95%)
HCV Genotype	3a	3	25	0.0203	0.424, 2.334
	1a	0	72		
Drug regimen	Daclatasvir/sofosbuvir \pm ribavirin	3	25	0.0203	0.424, 2.334
	Ledipasvir/sofosbuvir \pm ribavirin	0	72		
Severity of hemophilia	Severe	3	78	>0.999	0.201, 4.971
	Mild and moderate	0	19		
History of blood transfusion	Yes	3	82	>0.999	0.150, 6.669
	No	0	15		
History of plasma injection	Yes	3	65	0.5492	0.409, 2.441
	No	0	32		

Table 4. Characteristics of patients with occult HCV infection

Patient ID	Age (Year)	ALT Level IU/L	AST Level IU/L	History of blood transfusion	History of Plasma injection	Severity of hemophilia	HCV Genotype	DAAs regimes
		BT* AT**	BT AT				BT AT	
H74	52	87 22.3	92 18	Yes	Yes	Severe	3a 3a	DCV/SOF + RBV
H58	60	117 24	89 21	Yes	Yes	Severe	3a 3a	DCV/SOF + RBV
H29	52	65 25	55 20	Yes	Yes	Severe	3a 1a	DCV/SOF + RBV

*BT: Before treatment

**AT: After treatment

DCV: Daclatasvir, SOF: Sofosbuvir, RBV: Ribavirin

DISCUSSION

The first occult HCV infection was reported in HCV-positive patients with rapid spontaneous clearance of the virus, as well as in patients with an SVR due to interferon-based treatment with or without liver enzyme test [33, 34]. OCI is linked with liver fibrosis and progression of liver disease [35]. Therefore, it is required to investigate the occurrence of OCI in patients to avoid further complications. In the era of DAAs therapy for HCV infection, more than 90% of treatment was successful, and patients achieved SVR. However, a varied number of treated patients have experienced a late relapse. Thus, it was suggested that OCI is associated with a higher risk of late relapse [35]. Since the activation of OCI may be the origin of late relapse, herein, the presence of HCV RNA was studied in PBMCs and plasma samples of 100 hemophilia patients with negative serum HCV RNA.

Despite the great progress achieved in HCV treatment, chronic HCV infection is still a major health problem in high-risk groups such as hemophilia patients. Consuming blood-derived components in hemophilia patients can be a source of transfusion-transmitted infections, including HCV infection, resulting in liver failure in these patients [36]. In chronic HCV patients achieving SVR, HCV RNA could be detected in PBMCs [15, 16]. Thus, to determine the occurrence of OCI after treatment with DAAs regimens in Iranian hemophilia patients, we investigated the HCV RNA in their PBMCs samples. In various studies, the OCI prevalence was variable. For example, in Egypt, the prevalence of OCI among hemodialysis patients was 3.7% [37], while in Iran, 3.03% of PBMCs samples from similar patients were OCI positive [38]. To the best of our knowledge, this research is the first study about the presence of OCI in hemophilia patients treated with direct-acting antiviral regimens in Iran.

The seronegative OCI was documented with the implication of different HCV genotypes [39]. HCV genotypes including 1a, 2a, 3a, 3b and 4a were reported for OCI [21, 40]. Although HCV transmission by blood donation in developed countries is rare, further epidemiological studies should be done in various patient groups to investigate OCI as a global issue in order to modify necessary screening procedures.

In this study, 100 hemophilia participants were enrolled. All patients included in the study were negative for HBsAg, HIV and positive for HCV antibody. After 12 weeks of DAAs therapy and achieving SVR, HCV RNA was detected in 3% of PBMCs samples from hemophilia patients indicating the OCI despite sustained viral clearance. Thus, tracing HCV RNA in PBMCs is necessary to predict the response to antiviral therapy, including DAAs regimens. All three patients with OCI had HCV subtype 3a before DAAs treatment, and all were treated with DCV/SOF + RBV. Subsequently, this finding was in agreement with the findings of Wang *et al.* (2019), reporting that the onset of OCI is more frequent in patients with Genotype 3 [41].

Another important point of this study was that one of the participants (1%) showed a genotype difference between plasma and PBMCs samples. This was in accordance with

the mixed HCV genotypes because of multiple blood transfusion and plasma injection history [39-41]. Thus, PBMCs may present different subtypes other than plasma specimens indicating persistent infection. Risk groups, including hemophilia patients receiving large amounts of blood and clotting factors, might result in the mixed infection.

In conclusion, 3% of Iranian hemophilia patients (95% CI: 0.006-0.085) DAAs were positive for OCI, confirmed by positive results of the one-step RT-PCR method for PBMCs samples. Statistical data showed a significant association between OCI and genotype 3/drug regimens (p value=0.0203). One participant (1%) showed a genotype difference in plasma and PBMCs samples. This is in accordance with the mixed HCV genotype infection resulting in unresponsiveness to antiviral treatment and late relapse. Thus, it is essential to perform further studies with a large number of participants in the field of OCI on high-risk groups such as hemophilia patients. Generally, HCV infection is one of the treatable causes of liver disease, and investigating the prevalence and diagnosis of OCI in high-risk patients, including hemophilia patients, is critically important.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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