Prevalence of occult hepatitis C virus infection in beta-thalassemia major patients in Ahvaz, Iran

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Abstract
Occult hepatitis C virus infection (OCI) is defined by the presence of HCV RNA in peripheral blood mononuclear cells (PBMCs) and liver tissue cells despite the absence of HCV RNA in plasma. Currently, OCI is classified into two types: seropositive OCI (anti-HCV positive and serum HCV RNA negative) and seronegative OCI (anti-HCV and serum HCV RNA negative). Beta-thalassemia is described as a blood disorder that decreases the synthesis of hemoglobin. Repeated blood transfusion is the standard treatment for patients with beta-thalassemia major (BTM), and this increases the risk of exposure to infectious agents. The aim of this study was to investigate the prevalence of OCI among BTM patients. Plasma and PBMCs were collected from 90 BTM patients who were referred to Shafa Hospital in the city of Ahvaz and were screened for HCV antibody using a commercial ELISA kit as the first step. Next, nested RT-PCR was performed on extracts of plasma and PBMCs. HCV RNA from positive PBMCs was sequenced, the sequences were aligned, and a phylogenetic tree was constructed to determine their relationship to reference sequences retrieved from the GenBank database. Seventy-nine out of 90 patients (87.8%) were negative for HCV Ab (seronegative), while 11 patients (12.2%) were seropositive. HCV RNA was found in PBMCs of four patients (66.7%) who were negative for HCV Ab (seronegative) and two patients (33.3%) who were positive for HCV Ab (seropositive). HCV RNA was not detected in plasma samples from these six patients. Six out of 90 BTM patients (6.7%) had OCI. HCV genotyping revealed that all six patients were infected with HCV subtype 3a. We found a high frequency of OCI in BTM patients, which warrants more attention, considering the importance of this infection. Further studies are needed to determine the actual prevalence of OCI in BTM patients in Iran.

Introduction
Hepatitis C virus (HCV) is an enveloped positive single-stranded RNA virus belonged to the family Flaviviridae and genus Hepacivirus [1]. HCV has been classified into eight genotypes with 86 subtypes [2, 3]. HCV infection is transmitted through contact with infected blood, blood transfusion, drug injection, sexual intercourse, surgery, and the parenteral route [4, 5]. The course of infection can range from asymptomatic and acute to chronic liver diseases, such as fibrosis and hepatocellular carcinoma (HCC) [6]. It has been estimated that the global prevalence of HCV infection in adults is 2.5% [7]. Studies conducted on the general Iranian population found the estimated prevalence of HCV infection to be 0.5% [8].

Beta-thalassemia is defined as an inherited blood disorder affecting the synthesis of beta-globin chains in hemoglobin [9]. The disease is characterized by several complications, such as insufficient erythropoiesis and chronic hemolytic...
anemia [10]. Currently, regular blood transfusion is used as the first choice for the management of beta-thalassemia, and patients also receive iron-chelation therapy or bone marrow transplantation and supportive measures [11]. Blood transfusion carries a considerable risk of transmitting blood-transfusion-associated viral infections, such as HCV, hepatitis B virus (HBV), and human immunodeficiency virus (HIV) [12, 13].

HCV infection is a common cause of post-transfusion hepatitis in Iranian patients with beta-thalassemia major [14]. Iran has a large number of thalassemic carriers [15]. It has been reported that the prevalence of HCV among Iranian beta-thalassemia major patients is 19% [16]. In Iran, HCV genotype 1 is the predominant genotype, with a rate of 55%, followed by genotype 3 at 37%. Among patients with HCV genotype I, subtype 1a is the most predominant subtype, with a rate of 79%, followed by subtype 1b at 19% [17]. The HCV genotype is important for predicting a patient’s response to antiviral therapy. Therefore, genotype determination is the first step in the diagnosis and treatment of HCV infection. Moreover, the genotype may be an important factor contributing to the severity and aggressiveness of the resulting liver disease [18].

HCV is mainly hepatotropic, but peripheral blood mononuclear cells (PBMCs) are important extrahepatic sites of viral replication [19]. It has been shown in some patients that the HCV genotype in PBMCs differs from that in the plasma [20–24]. The genotype of HCV RNA at extrahepatic sites is a major prognostic factor for the success of treatment [19, 24]. PBMCs are important sites of HCV replication after treatment, at least with interferon treatment [20, 25]. It has been argued that the main cause of relapse or reinfection with HCV after liver transplantation is viral replication at extrahepatic sites [25].

Occult HCV infection (OCI) is a recently recognized form of HCV infection that is characterized by the presence of HCV RNA in the liver tissue or PBMCs in the absence of HCV RNA in plasma, with or without HCV antibodies (anti-HCV). There are two clinical forms, based on the presence of anti-HCV Abs: seropositive with normal liver enzyme levels and seronegative with high liver enzyme levels. Although the most reliable method for diagnosing OCI in all patients is detection of the HCV genome in the liver, the detection of HCV RNA in PBMCs is an alternative approach when a liver biopsy is not available. The current method for diagnosing HCV in Iran is the detection of anti-HCV antibodies by ELISA and the detection of HCV RNA by nucleic acid amplification test (NAT) in plasma, which is unable to detect OCI [8, 26, 27].

Patients with OCI are potentially infectious, and the infection may lead to the development of cryptogenic liver cirrhosis, fibrosis, and HCC. Repeated transfusion and a lack of molecular screening tests of blood donations for the presence of HCV RNA in PBMCs could increase the likelihood of OCI [28]. In this study, we investigated the prevalence of OCI and risk factors for this infection in Iranian beta-thalassemia major patients in the city of Ahvaz. Hence, it is critical to screen patients with beta-thalassemia undergoing blood transfusion to detect OCI cases.

Materials and methods

Ethical issues

The current research was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, with the registration number OG-9734 and ethical code IR.AJUMS.REC.1397.745. Patients were informed of the present research and written consent was obtained before their enrollment.

Study population

A total of 90 Iranian patients with beta-thalassemia major participated in this cross-sectional study between January 2018 and March 2019, at Shafa Thalassemia Clinic. The inclusion criteria were as follows: 1) the patients were residents of Ahvaz, 2) they had the mental capacity to give written informed consent, and 3) they were patients with beta-thalassemia who regularly received at least one unit of blood per month. The exclusion criteria were as follows: 1) the patients did not agree to participate in the study, 2) they were positive for HIV antibody, 3) they were hemophilic or had other types of hemolytic anemia, such as α-thalassemia, sickle cell anemia, or spherocytosis. Patients’ medical profiles were checked for demographic information, clinical history, and laboratory characteristics.

Collection and preparation of the specimens

Approximately 7 mL of peripheral blood was collected from the patients in a sterile EDTA-containing vacutainer tube. The plasma was separated by centrifugation at 2500 rpm for 5 min and stored at -80°C until testing. PBMCs were isolated by Ficoll-Hypaque (Lymphodex, Inno-Train, Germany) density gradient centrifugation, and the pellets of PBMCs were washed three times with phosphate-buffered saline (pH 7.3 ± 0.1). Cells were kept in 200 µl of RNA Later solution (Ambion, Austin, TX) and stored at -80°C until further examination.

Serological and biochemical tests

Anti-HCV antibody in plasma samples was detected using a third-generation commercial ELISA kit (DIAPRO,
Diabetic. BioProbes Srl. Milano, Italy) according to the manufacturer’s instructions. All patients were tested for alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), cholesterol (Chol), and lymphocytes.

**Extraction of viral RNA**

Viral RNA from both plasma (200 µl) and the PBMC pellet (about 3-5 x 10^6 cells) was extracted using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The purity of viral RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and RNA samples had an optical density (A260/A280) ratio of ≥1.90, indicating their purity [29].

**Detection of HCV RNA by RT-nested PCR**

Complementary DNA (cDNA) was prepared using a cDNA synthesis kit (Yekta Tajhiz, Iran). Briefly, 1 µl of 50 µM random hexamer and 13.4 µl of diethyl pyrocarbonate (DEPC)-treated water were mixed to make a mixture of 0.5 µg of RNA template. The mixture was incubated at 72°C for 5 minutes and cooled quickly on ice. Then, 4 µl of 5X reaction buffer was mixed with 1 µl of deoxynucleotide triphosphate (dNTPs) (10 mM), 0.5 µl of RNase inhibitor (20 units), and 1 µl of reverse transcriptase (200 units). The mixture was added to the previous mixture and incubated at 37°C for 60 minutes. Finally, the reaction was halted by heating at 70°C for 5 minutes. The prepared cDNA was stored at -80°C for further testing.

To detect HCV RNA, the 5’ untranscribed region (5’-UTR) of the HCV genome was amplified by reverse transcription nested polymerase chain reaction (RT-nested PCR) using specific primers. The sequences of the primers were as follows: BKP-7, CACTCCCTGTGAGGAACTACTGTC (nucleotides 38 to 62) as the outer sense primer; BKP-8, ATGGTGACAGGCTCTAGAGCCTTC (nucleotides 319 to 343) as the outer antisense primer; BKP-9, TTCACG CAGAAAGCCTCTAGACCTTC (nucleotides 63 to 87) as the inner sense primer; and BKP-10, GGCACCTGCAAA GCACCTATACGG (nucleotides 292 to 314) as the inner antisense primer [30].

RT-nested PCR amplification for the first round was carried out in a total volume of 25 µl consisting of 12.5 µl of PCR master mix, 1 µl of each forward and reverse primer, 2 µl of cDNA, and 8.5 µl of distilled water. In the second round, 1 µl of the product of the first round was used as a template, and 9.5 µl distilled water was added to reach the final 25 µl volume. The first- and second-round reactions were performed in a thermocycler (Peqlab, Germany) with the following thermal program: initial denaturation at 94°C for 5 min; then 35 (first round) or 30 (second round) cycles of 94°C for 30 s, 62°C for 45 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. An amplicon of 252 bp length was detected by electrophoresis in a 2% agarose gel after the second round of PCR. RT-nested PCR was then performed on PBMC extracts from patients in negative results at the previous step. The detection of a second-round PCR product in a 2% agarose gel indicated that the patient was positive for OCI results. To confirm this, HCV RNA was amplified by RT-nested PCR using two primer sets from the core region [31].

**RT-nested PCR for the core region**

Samples that were positive by PCR targeting the 5’-UTR region were tested again, this time targeting the core region of the HCV genome by RT-nested PCR. The following specific primers were used: SC2 (GGGGTTTCTCGTAG CCGTGCACCATG), AC2 (GAGGGKTARTACCCCATGAGRTC), S7 (AGACCGTGACCATGACGAC), and 584 (CCCATAGGTCCGGCAARC) [32]. Two µl of the template in the same volume of PCR reaction mixture as described above was subjected to thermocycling for 30 and 25 cycles for the first and second rounds, respectively. The cycling conditions were as follows: 94°C for 4 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and final elongation at 72°C for 7 min. The expected PCR products for the outer set and the inner set were 500 bp and 420 bp in length. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with DNA Safe Stain, and observed under ultraviolet light.

**HCV genotyping and subtyping by nucleotide sequencing and phylogenetic analysis**

The purified products from the second round of PCR amplification were sequenced in both the forward and reverse directions using an ABI 3730 XL DNA sequencer (BIONEER Custom Sequencing Service). Analysis and alignment of forward and reverse sequences were performed using SnapGene sequence alignment editor version 3.2.1. The sequences were deposited in the GenBank database under the accession numbers MN401419-MN401424. For phylogenetic analysis, MEGA X software was utilized. The maximum-likelihood method with the Tamura-Nei model was used for the construction of a phylogenetic tree and identification of HCV genotypes. One thousand bootstrap replicates were performed to calculate the confidence level for each branch of the tree.
Statistical analysis

SPSS software version 22 (SPSS Inc, Chicago, IL, USA) was used for data analysis. Data were analyzed using the Mann-Whitney U-test and Fisher’s exact test. Odds ratios and confidence intervals were used to compare differences between groups. A P-value lower than 0.05 was considered statistically significant.

Results

This cross-sectional study included 90 patients with beta-thalassemia major. Serological tests revealed that 79 of the 90 subjects (87.8%) were negative for anti-HCV Ab, while 11 participants (12.2%) were positive. RT-nested PCR targeting the HCV 5’-UTR revealed that 24 (26.7%) of the subjects were positive for HCV RNA in their plasma and were therefore excluded from the study due to overt HCV infection. As shown in Table 1, the remaining 66 (72.6%) patients, 35 (53%) of which were male and 31 (47%) of which were female, with a mean age of 23.54 ± 8.08 (range, 4-47) years, were negative for HCV RNA in their plasma. Six of these 66 patients (9.1%) were positive for HCV RNA in their PBMCs (including four who were negative for HCV Ab and two who were positive for HCV Ab), demonstrating OCI in these patients. Overall, six out of 90 patients (6.7%) had OCI. Two OCI patients (33.3%) were seropositive (positive for HCV Ab and negative for serum HCV RNA) and four (66.7%) were seronegative (negative for both HCV Ab and serum HCV RNA) (Fig. 1). No significant association was found between AST, ALT, TG, cholesterol, or lymphocyte counts and the presence or absence of OCI. However, the patients with OCI had a higher mean ALT (62.67 ± 26.77) and cholesterol (107 ± 1.41) levels than the OCI-negative patients (ALT, 58.24 ± 57.58; cholesterol, 102.61 ± 25.02), but like in other studies,

| Parameter                      | Total            | Negative HCV RNA in PBMCs | Positive HCV RNA in PBMCs | Odds ratio (95% CI) | P-value |
|--------------------------------|------------------|---------------------------|---------------------------|---------------------|---------|
| Age                            | 23.54±8.08       | 23.23±7.54                | 26.50±12.72               | 1.28 (0.76, 2.16)   | 0.274   |
| (for 5-year increase)          | 24 (10)          | 23 (8.5)                  | 31.5 (21.5)               |                     |         |
| Gender                         |                  |                           |                           |                     |         |
| Male                           | 35 (53%)         | 32 (91.4%)                | 3 (8.6%)                  | Reference           | 1       |
| Female                         | 31 (47%)         | 28 (90.3%)                | 3 (9.7%)                  | 1.14 (0.21, 6.12)   |         |
| Laboratory parameters          |                  |                           |                           |                     |         |
| AST (IU/L)                     | 53.18±38.64      | 54.43±40.41               | 42.50±15.71               | 1.43 (0.53, 3.89)   | 0.771   |
| (for 30 unit decrease)         | 42 (34.5)        | 41 (35)                   | 45 (28.8)                 |                     |         |
| ALT (IU/L)                     | 58.70±55.01      | 58.24±57.58               | 62.67±26.77               | 1.03 (0.68, 1.56)   | 0.325   |
| (for 30 unit increase)         | 45 (58)          | 43 (59)                   | 58.50 (39.3)              |                     |         |
| TG (mg/dl)                     | 133.48±69.26     | 138.04±69.57              | 72±18.38                  | 2.18 (0.67, 7.07)   | 0.099   |
| (for 20 unit decrease)         | 109 (83)         | 126 (83)                  | 72 (-)                    |                     |         |
| Cholesterol (mg/dl)            | 102.90±24.17     | 102.61±25.02              | 107±1.41                  | 1.15 (0.35, 3.73)   | 0.777   |
| (for 20 unit increase)         | 103.5 (34.3)     | 99.50 (36.8)              | 107 (-)                   |                     |         |
| Lymphocyte                     | 44.62±8.90       | 44.71±8.58                | 43.82±12.61               | 1.13 (0.38, 3.38)   | 0.619   |
| (for 10 percent decrease)      | 43.4 (10.55)     | 43.55 (10.50)             | 40 (20.55)                |                     |         |
| Anti-HCV Ab                    |                  |                           |                           |                     |         |
| Negative                       | 60 (90.9%)       | 56 (93.3%)                | 4 (6.7%)                  | Reference           | 0.088   |
| Positive                       | 6 (9.1%)         | 4 (66.7%)                 | 2 (33.3%)                 | 7 (0.97, 50.57)     |         |
| HBS Ag                         |                  |                           |                           |                     |         |
| Negative                       | 64 (97%)         | 58 (90.6)                 | 6 (9.4%)                  | -                   | -       |
| Positive                       | 2 (3%)           | 2 (100%)                  | 0                        |                     |         |
| Epidemiological parameters     |                  |                           |                           |                     |         |
| Blood transfusion              | 272.45±99.15     | 269.39±94.44              | 301±143.97                | 1.16 (0.79, 1.72)   | 0.297   |
| No. (for 50-unit increase)     | 276 (120)        | 274 (105.8)               | 354 (244.5)               |                     |         |
| Splenectomy history            |                  |                           |                           |                     |         |
| No                             | 48 (78.4%)       | 44 (91.7%)                | 4 (8.3%)                  | Reference           | 0.61    |
| Yes                            | 14 (22.6%)       | 12 (85.7%)                | 2 (14.3%)                 | 1.83 (0.30, 11.24)  |         |
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these differences were not statistically significant. The biochemical characteristics of all the patients included in the study are shown in Table 1.

HCV genotyping was carried out by sequencing the HCV 5'-UTR for all of the positive OCI patients. Analysis of the results indicated that all six patients had HCV genotype 3a (Table 2). Complete information about beta-thalassemia patients with OCI is shown in Table 2.

The nucleotide sequences obtained by sequencing of the HCV 5'-UTR PCR products were submitted to the GenBank database with accession numbers MN401419 to MN401424 (PBMC samples of six patients with OCI). Phylogenetic analysis of these six sequences and a corresponding reference sequence obtained from GenBank (NC-009824) demonstrated that all six OCI isolates from Ahvaz were subtype 3a (Fig. 2).

Discussion

HCV infection is one of the most important causes of mortality in BTM patients, as it can lead to hepatic failure in these patients [33]. In the present work, we assessed the prevalence of OCI among Iranian subjects with beta-thalassemia major referred to Shafa Hospital in the city of Ahvaz who had negative results for HCV RNA in their serum samples, with or without the presence of anti-HCV Abs. As shown in Table 1, there was no significant association between the laboratory parameters and the presence or absence of OCI, which is consistent with several previous reports. Kahyesh-Esfandiary and coworkers and Bastani et al. in another study in Iran showed that there was no significant association between ALT, AST, and the presence of OCI in beta-thalassemia major patients [27, 33]. Ayadi et al. reported that beta-thalassemia major patients with OCI had normal levels of ALT and AST,
although AST and ALT levels were near the upper limits in one and two cases, respectively, and triglyceride and LDL levels were higher in patients with OCI than in non-OCI patients [32].

In the present study, RT-nested PCR showed that, six out of 90 (6.7%) beta-thalassemia patients undergoing blood transfusion had OCI, two of which (33.3%) were seropositive and four (66.7%) of which were seronegative, based on anti-HCV Ab ELISA results. On the other hand, the risk of OCI among seropositive patients was found to be seven times that of seronegative patients (OR = 7). Although this difference is not statistically significant, it is borderline ($p = 0.088$) (Table 1). Furthermore, sequence analysis revealed that all six patients had HCV genotype 3a (Table 2). The sequences obtained in this project were 100% identical to that of strain NCV1/PK1 from Pakistan (JN588558) (Fig. 2).

These findings are in accordance with the results of other studies investigating the OCI status among Iranian beta-thalassemia major patients, which indicated a prevalence rate of 3.3%-6.3% for OCI among the population [28, 33, 34]. Bastani and colleagues found that six (5.7%) out of 106 patients with beta-thalassemia major evaluated in their study had OCI. The authors used PBMC samples to detect OCI cases by RT-nested PCR. Moreover, HCV subtyping indicated that three, two, and one of the subjects had OCI caused by HCV genotype 1b, 3a, and 1a, respectively [28]. Kahyesh-Esfandiary et al. analyzed genomic HCV RNA in 48 PBMC specimens collected from Iranian patients with negative plasma HCV RNA and found that three (6.3%) of the subjects were also positive for OCI by RT-nested PCR and that genotypes 1a and 3a were the most common subtypes [34]. Similarly, in another study from Iran, Ayadi and coworkers showed that six (3.3%) out of 181 beta-thalassemia patients had HCV RNA in their PBMC samples and were thus classified as OCI patients. Furthermore, HCV genotypes 1b (three subjects), 1a (two subjects), and 3a (one subject) were identified in that study [33]. As HCV genotyping results from the above-mentioned studies and the present work demonstrate, the HCV genotypes found in OCI patients represent the most common HCV subtypes in Iran: 1a, followed by 3a and 1b [35]. Genotyping is important for planning HCV treatment. The response to treatment is different for the different genotypes, and a specific therapeutic regimen is therefore needed for each genotype. Determination of the HCV genotype is thus essential for monitoring therapeutic regimens, improving local control programs, and eventually producing an effective vaccine [36]. As treatment of HCV infection has evolved, genotype 3 virus infections have become the most difficult to treat, with more rapid progression to fibrosis and cirrhosis than with the other genotypes. HCV genotype 3 infection is associated with a higher prevalence of severe steatosis and increased risk of HCC and mortality from all causes [18].
Previous investigations have shown that the frequency of HCV infection among Iranian patients with beta-thalassemia major varies from 7% to 64% [34, 37], which is in agreement with the overt infection results from the present work, showing that 24 out of 91 (26.7%) subjects had HCV infection. On the other hand, it is known that approximately 70% of OCI patients may be positive for HCV RNA in their PBMCs [38]. Thus, OCI may have a higher prevalence rate among Iranian beta-thalassemia major patients. Regarding the significant mortality rate of HCV infection in thalassemic patients, OCI should be given more consideration in this group. Indeed, it seems that the likelihood of OCI should be considered not only in thalassemic patients but also in other individuals who are at an increased risk of blood transfusion-associated viral infections.

Conclusion

Our study showed a prevalence rate of 6.7% OCI without evidence of the presence of HCV RNA in the plasma in beta-thalassemia patients. Considering the serious complications and the clinical importance of OCI in beta-thalassemia patients, sensitive diagnostic methods for identifying HCV RNA in PBMCs should be implemented for all thalassemia patients when a liver biopsy is not available. Further studies with a larger number of samples are needed to obtain a better estimate of OCI and to evaluate the significance and repercussions of OCI in patients with beta-thalassemia major.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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