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Prevalence of Occult Hepatitis C Virus Infection in Beta-Thalassemia Major Patients in Ahvaz, Iran

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Prevalence of Occult Hepatitis C virus infection in beta-Thalassemia major patients in Ahvaz, Iran

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Running Title: Occult Hepatitis C Infection among beta-thalassemia major Patients

Abstract

Background: 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, which decreases the synthesis of hemoglobin. Repeated blood transfusion is the standard treatment for patients with beta-thalassemia major (BTM) that increases the risk of exposure to infectious agents. This study aimed to investigate the prevalence of OCI among BTM patients.

Materials and Methods: plasma and PBMCs were collected from 90 BTM patients and screened for HCV antibody using the ELISA kit commercially as the first step. Then nested-RT PCR was performed on extractions of plasma and PBMC. Positive samples of HCV RNA from PBMCs were sequenced and aligned to construct the HCV phylogenetic tree to assess the homology of sequences compared to the reference sequences retrieved from GenBank.

Results: Seventy-nine out of 90 cases (87.8%) indicated negative results for HCV Ab (seronegative), while 11 patients (12.2%) were seropositive. HCV RNA was found in PBMCs samples of four patients (66.66%) with negative HCV Ab (seronegative) and two patients (33.3%) with positive HCV Ab (seropositive). HCV RNA was not detected in plasma samples of these six patients. Overall six out of 90 patients (6.7%) had OCI. HCV genotyping revealed that all six patients infected with HCV subtype 3a.

Conclusion: We indicated the high frequency of OCI in BTM patients. Nevertheless, more attention is warranted, considering the importance of this infection. Also, further studies are necessary to determine the actual prevalence of OCI among BTM patients in Iran.

Key words: Occult hepatitis C virus infection, Beta-Thalassemia major, HCV Ab, prevalence

Introduction

Hepatitis C Virus (HCV) is an enveloped positive single-stranded RNA virus belonged to the Flaviviridae family and Hepacivirus genus (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 HCV infection prevalence 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 also patients receive iron-chelation therapy, or bone marrow transplantation and supportive measures (11). Therefore, blood transfusion carries a considerable risk of acquiring blood transfusion-associated viral infections, such as hepatitis C virus, hepatitis B virus (HBV), and also human immunodeficiency virus (HIV) (12, 13).

HCV infection is the common cause of post-transfusion hepatitis between Iranian patients with beta-thalassemia major (14). Iran has high levels 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 was the predominant genotype with a rate of 55% followed by genotype 3 at 37%. Among patients with HCV genotype 1, subtype 1a was the most predominant subtype with a rate of 79%, followed by subtype 1b at 19% (17).

HCV is mainly hepatotropic, but peripheral blood mononuclear cells (PBMCs) are the extrahepatic sites of viral replication (18). It has been shown in some patients; the HCV genotype in PBMC is different from that in the plasma (19-23). The genotype of HCV RNA in extrahepatic sites is a major prognostic factor for the HCV infection treatment process (18, 23). PBMCs are a favorite site for replicating HCV after treatment, at least with interferon treatment (19, 24). It is argued that the main cause of relapse or reinfection of HCV infection after liver transplantation is HCV replication in extrahepatic sites (24).

Occult HCV infection (OCI) is a new 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). 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 detecting the HCV genome in the liver, the detection of HCV-RNA in PBMC is an alternative approach when a liver biopsy is not available. The current method for diagnosing HCV in Iran is detecting anti-HCV antibodies by ELISA and the detection of HCV RNA by Nucleic acid amplification tests (NAT) in plasma, which is unable to detect OCI (8, 25, 26).

Patients with OCI are potentially infectious, and the infection may be involved in 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 (27). This research aims to determine the prevalence of OCI and to detect risk factors for this infection among Iranian beta-thalassemia major patients. Hence, it is critical to screen patients with beta-thalassemia undergoing blood transfusion to detect OCI cases.

Materials and methods

Ethical issues

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

Study population

In this cross-sectional analysis performed between January 2018 and March 2019, a total of 90 Iranian patients with beta-thalassemia major referred to Shafa Thalassemia Clinic enrolled. Inclusion criteria included: 1) patients living in Ahvaz city, 2) patients with the mental capacity to give written informed consent, and 3) patients with beta-thalassemia who regularly receive at least one unit of blood per month. The exclusion criteria included: 1) patients who disagreed to follow the study, 2) patients positive for HIV antibody, 3) Hemophilic patients, and patients with other types of hemolytic anemia, such as α thalassemia, sickle cell anemia, and spherocytosis. Patients'

medical profiles were checked for demographic information, clinical history, and finding laboratory characteristics.

Collection and preparation of the specimens

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

Serological and biochemical tests

The Anti-HCV antibody in plasma samples was tested by a third-generation commercial ELISA kit (DIAPRO, Diagnostic, BioProbes Srl. Milano. Italy) according to manufacturer's instructions. All patients were tested for alanine aminotransferase (ALT), aspartate aminotransferase (AST), Triglyceride (TG), Cholesterol (Chol), and Lymphocyte.

Extraction of viral RNA

Viral RNA from both plasma (200 µl) and pellet of PBMCs (about 3-5 $\times$ 10^6 cells) were extracted by a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions. The purity and concentration of viral RNA were measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and RNA samples had an concentration (A260/A280) ratio of $\geq$190 (28).

Detection of HCV RNA by RT-Nested PCR

The complementary DNA (cDNA) was prepared using a cDNA synthesis kit (Yekta Tajhiz, Iran). In short, one µl of 50 µM random hexamer and 13.4 µl of diethyl pyrocarbonate (DEPC) water were mixed to make a mixture 0.5 µg of RNA template. The mixture was incubated at 72°C for 5 minutes and cooled quickly on the ice. Then, four µl of 5X reaction buffer was mixed with one µl of deoxynucleotide triphosphate (dNTPs) (10 mM), 0.5 µl of the RNase inhibitor (20 units) 1µl , and 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 deposited at -80 ° C for further testing.

To detect HCV RNA, the 5′-untranslated region (5′-UTR) of the HCV genome was amplified by reverse transcriptase nested polymerase chain reaction (RT-nested PCR) using specific primers. The sequences of the outer primers were BKP-7, CACTCCCCTGTGAGGAACTACTGTC (nucleotides 38 to 62) as the outer sense, BKP-8, ATGGTGCACGGTCTACGAGACCTCC (nucleotides 319 to 343) as the outer anti-sense; BKP-9,TTCACGCAGAAAGCGTCTAGCCATG (nucleotides 63 to 87) as the inner sense; BKP-10, GCGCACTCGCAAGCACCCTATCAGG (nucleotides 292 to 314) as the inner anti-sense primer (29).

RT-Nested PCR amplification for the first round was carried out with a total of 25 µl consisting of 12.5 µl PCR master mixture, one µl of each forward and reverse primer, two µl of cDNA, and 8.5 µl of distilled water. In the second round, one µl 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 rounds of RT-Nested PCR reaction mixture was subjected to thermocycler (Peqlab, Germany) with the following thermal program: initial denaturation at 94ºC for 5 min; and then 35 cycles of 94ºC/ 30″, 62ºC/ 45″, 72ºC/ 30″, and a final extension at 72ºC for 10 min. The amplicon of 252 bp length

was detected by electrophoresis on 2% agarose gel as second-round PCR products. Then RT-Nested PCR was performed on PBMC extraction of patients with negative results at the previous step. The second-round PCR products on 2% agarose gel, identified as positive OCI results. To confirm positive OCI patients, HCV RNA was amplified using RT-Nested PCR with two primers set from the core region (30).

RT-Nested PCR for the core region

The positive samples for the 5'-UTR region were again tested for the HCV core region of the HCV genome by RT-Nested PCR. The following specific primers Including, SC2: GGGAGGTCTCGTAGACCGTGCACCATG, AC2: GAGMGGKATRTACCCATGAGRTCGC, S7: AGACCGTGCACCATGAGCAC, and 584: CCCATGAGGTCGGCRAARC were used (31). Two µl of the template with the same volume of PCR reaction mixture as described above was subjected to thermocycler for 30 cycles. The cycling conditions were achieved as follows: 94^0C for 4 min; 30 cycles at 94^0C for 1min, 45^0C for 1 min, 72^0C for 2 min; and final elongation at 72^0C for 7 min. The expected PCR product for the outer set and the inner set was 500bp and 420 bp. PCR product was subjected to electrophoresis on a 2% agarose gel, stained with DNA safe stain, and observed under ultraviolet light.

HCV genotyping/sub-typing using Nucleotide Sequencing and phylogenetic analysis

The purified products from the second round of PCR amplification in both the forward and reverse directions were sequenced using an ABI 3730 XL DNA sequencer (BIONEER's Custom Services - Sequencing Service). Analysis and alignment of forward and reverse sequences were performed by SnapGene sequence alignment editor version 3.2.1. The sequences were deposit in the GenBank database under the accession numbers: MN401419- MN401424. For phylogenetic analysis, MEGA X software was utilized. The Maximum likelihood method with Tamura Nei-model was

used for the construction phylogenetic tree and identification of HCV genotypes. 1000 bootstrap replicates performed the confidence level of evolutionary distances.

Statistical analysis

SPSS software version 22 (SPSS Inc, Chicago, IL, USA) was used for data analysis. Analyses of data were performed using Mann-Whitney U-test and fisher's exact test. Furthermore, Odds Ratio and Confidence Intervals were used for comparing differences between groups. The P-value lower than 0.05 was considered statistically significant.

Results

The existing cross-sectional research enrolled 90 patients who suffered from beta-thalassemia major. Serological experiments revealed that 79 of 90 subjects (87.8%) were negative for anti-HCV Ab, while 11 participants (12.2%) were positive. The RT-Nested PCR results for the HCV 5'-UTR revealed that 24 cases (26.7%) in their plasma were positive for HCV-RNA, and hence, excluded from the study owing to overt HCV infection. As Table-1 shows, of the remaining 66 (73.33%) patients, in their plasma were negative for HCV-RNA. The findings also revealed that six out of 66 remaining patients (9.1%) were positive for HCV-RNA in their PBMCs (including four cases with negative HCV Ab, two cases with positive HCV Ab), demonstrating OCI in these patients. Overall six out of 90 patients (6.7%) had OCI. In other words, two individual of OCI patients (33.3%) were seropositive (positive for HCV Ab and negative for serum HCV-RNA) while four individual of OCI patients (66.66%) were seronegative (negative for both HCV Ab and serum HCV-RNA) (Figure 1). The biochemical characteristics of all the patients included in the study are shown in Table 1.

HCV genotyping was carried out via the HCV 5'-UTR sequencing for all 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 are shown in table-2. The nucleotide sequences obtained from the sequencing of the HCV 5'-UTR were recorded to GenBank with accession numbers MN401419 to MN401424 (PBMCs samples of six patients with OCI). Phylogenetic analysis of these six sequences and corresponding sequence recovered from GenBank (NC-009824) demonstrated that all six OCI isolated from Ahvaz were subtype 3a (Figure-2).

Table-1. Iranian beta-thalassemia patients demographic and clinical data

Parameter	Total	Negative HCV RNA	Positive HCV RNA	Odd ratio	*P*.value

		in PBMC(group 1)	in PBMC(group2)	(95% CI)	
Age (For 5 year increase)	23.54±8.08 24 (10)	23.23± 7.54 23 (8.5)	26.50±12.72 31.5 (21.5)	1.28 (0.76, 2.16)	0.274
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) (For 30 unit decrease)	53.18±38.64 42 (34.5)	54.43±40.41 41(35)	42.50±15.71 45 (28.8)	1.43 (0.53, 3.89)	0.771
ALT (IU/L) (For 30 unit increase)	58.70±55.01 45 (58)	58.24±57.58 43 (59)	62.67±26.77 58.50 (39.3)	1.03 (0.68, 1.56)	0.325
TG (mg/dl) (For 20 unit decrease)	133.48±69.26 109 (83)	138.04±69.57 126 (83)	72±18.38 72 (-)	2.18 (0.67, 7.07)	0.099
Cholesterol (mg/dl) (For 20 unit increase)	102.90±24.17 103.5 (34.3)	102.61±25.02 99.50 (36.8)	107±1.41 107(-)	1.15 (0.35, 3.73)	0.777
Lymphocyte (For 10 percent decrease)	44.62±8.90 43.4 (10.55)	44.71±8.58 43.55 (10.50)	43.82±12.61 40 (20.55)	1.13 (0.38, 3.38)	0.619
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 No. (for 50 unit increase)	272.45± 99.15 276 (120)	269.39±94.44 274 (105.8)	301±143.97 354 (244.5)	1.16(0.79, 1.72)	0.297
Splenectomy History					
No	48 (78.4%)	44 (91.7%)	4 (8.3%)	Reference	0.610
Yes	14 (22.6%)	12 (85.7%)	2 (14.3%)	1.83 (0.30, 11.24)	

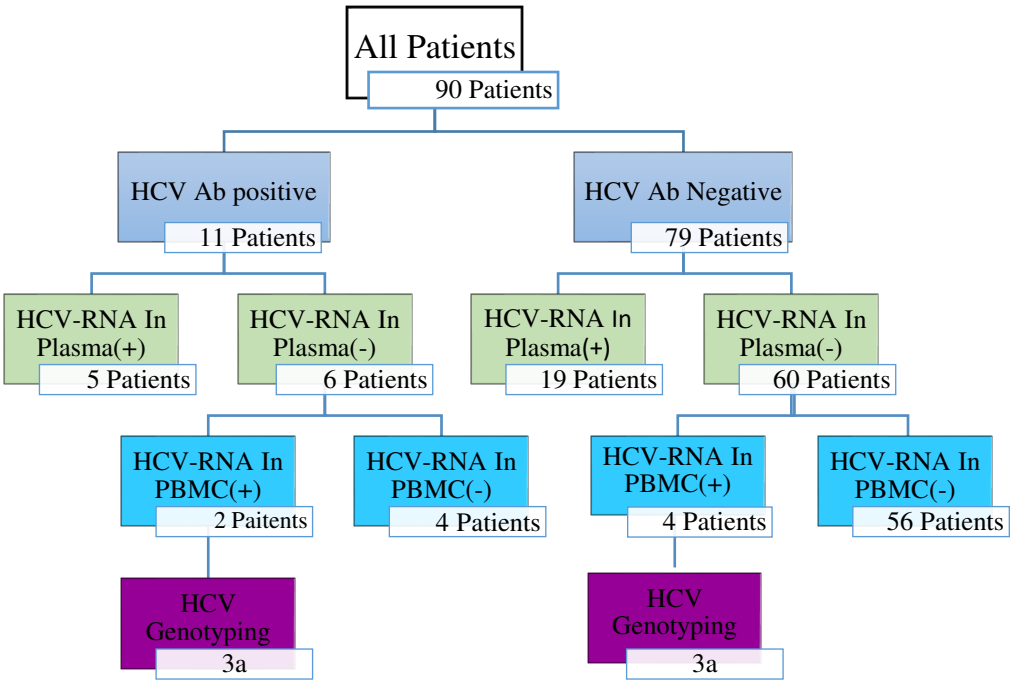

Figure 1. All the results obtained by serological and molecular experiments in this research.

Table-2. The characteristics of beta-thalassemia major with occult HCV infection

Patient No.	Gender/Age	Blood type	Transfusion No.	ALT (IU/L)	AST (IU/L)	HCVAb in serum	HCV-RNA in plasma	HCV-RNA in PBMC[e]	HCV genotype in PBMC
P 11	M [b] /35	AB-	414	59	53	N[c]	N	P[d]	3a
P 13	M [b] /5	O+	54	58	44	N	N	P	3a
P 16	F [a] /18	B+	210	100	46	N	N	P	3a
P 19	M [b] /36	O-	384	82	29	P	N	P	3a
P 25	F [b] /37	A+	420	21	20	P	N	P	3a
P 31	F [a] /28	A+	324	56	63	N	N	P	3a

[a] Female, [b] Male, [c] Negative, [d] Positive, [e] Peripheral blood mononuclear cells

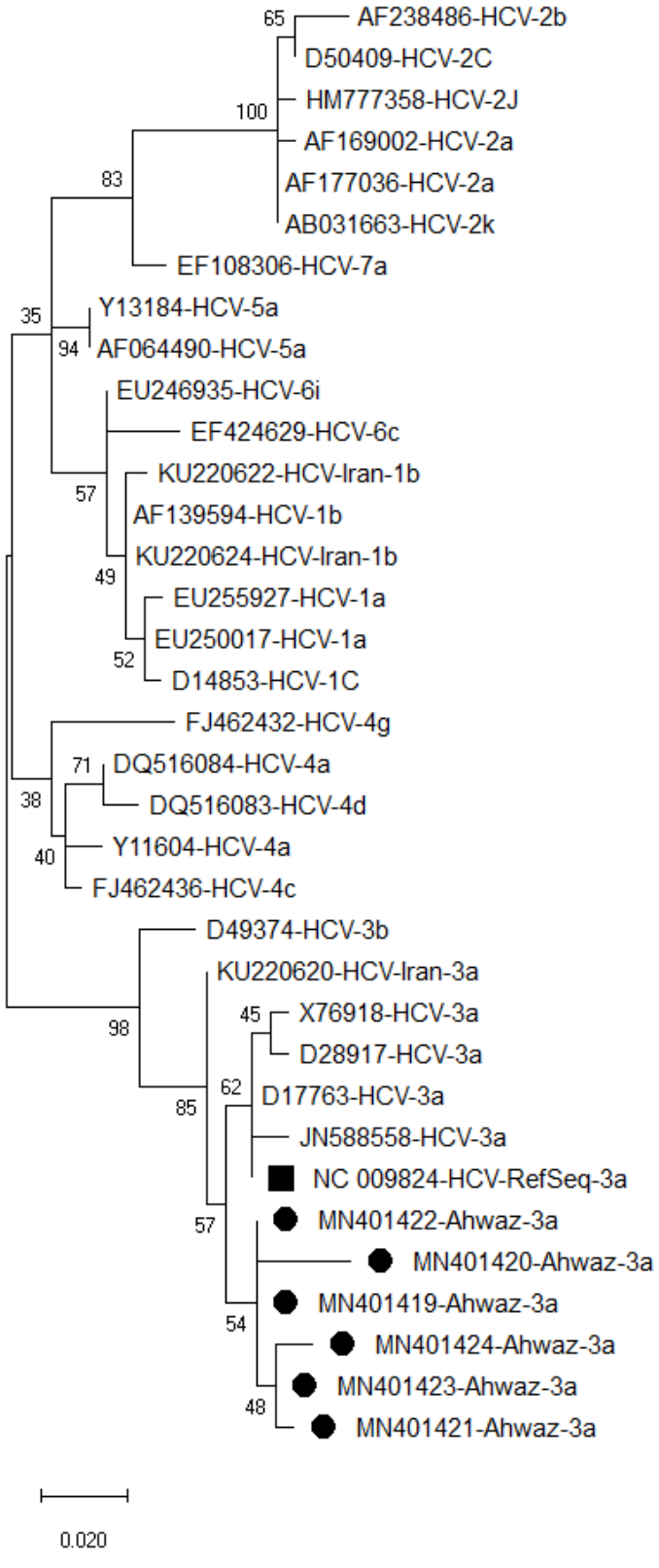
AF238486-HCV-2b
D50409-HCV-2C
HM777358-HCV-2J
AF169002-HCV-2a
AF177036-HCV-2a
AB031663-HCV-2k
EF108306-HCV-7a
Y13184-HCV-5a
AF064490-HCV-5a
EU246935-HCV-6i
EF424629-HCV-6c
KU220622-HCV-Iran-1b
AF139594-HCV-1b
KU220624-HCV-Iran-1b
EU255927-HCV-1a
EU250017-HCV-1a
D14853-HCV-1C
FJ462432-HCV-4g
DQ516084-HCV-4a
DQ516083-HCV-4d
Y11604-HCV-4a
FJ462436-HCV-4c
D49374-HCV-3b
KU220620-HCV-Iran-3a
X76918-HCV-3a
D28917-HCV-3a
D17763-HCV-3a
JN588558-HCV-3a
NC 009824-HCV-RefSeq-3a
MN401422-Ahwaz-3a
MN401420-Ahwaz-3a
MN401419-Ahwaz-3a
MN401424-Ahwaz-3a
MN401423-Ahwaz-3a
MN401421-Ahwaz-3a
65
100
83
35
94
57
49
52
71
38
40
98
45
62
85
57
54
48
0.020

Figure 2. The phylogenetic tree based on the nucleotide sequences of HCV 5'-UTR region obtained from PBMC samples from participants who suffer from beta-thalassemia major with OCI. The tree was constructed using MEGA X based on maximum likelihood under the Tamura Nei-model. 1000 bootstrap replicates measured the tree's precision. In black circles the isolations obtained in this study are shown (●).In the black square, the reference sequence is shown.

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 (32). The present work assessed the prevalence of OCI among the Iranian subjects with beta-thalassemia major who had negative results for HCV RNA in their serum samples with or without the presence of anti-HCV Abs. Regarding the laboratory parameters AST, ALT, TG, Cholesterol, and Lymphocyte, there was no significant association between the parameters and the presence or absence of OCI. However, the patients with OCI have a higher mean of ALT (62.67± 26.77) and Cholesterol (107± 1.41) than the OCI-negative ones (ALT: 58.24± 57.58, Cholesterol: 102.61± 25.02) but like other studies, it has been no association statistically. There are several reports on the association between liver tests and serum lipids in the presence of OCI. In this way, Kahyesh-Esfandiary and co-workers and Bastani et al in another study from Iran showed there was no significant association between ALT, AST, and the presence of OCI among beta thalassemia major patients (33, 27). Ayadi et al reported bet - thalassemia major patients with OCI had normal levels of ALT and AST, although one and two cases were at the upper limits of AST and ALT, respectively and triglyceride and LDL levels were higher in patients with OCI, compared with non-OCI patients (32).

As the RT-nested PCR from the present study shows, overall, six out of ninety (6.7%) beta-thalassemia patients undergoing blood transfusion had OCI, of which two (33.3%) and four (66.66%) cases were seropositive and seronegative, respectively, based on the anti-HCV Ab findings. On the other hand, the risk of OCI among seropositive persons is seven times that of seronegative ones (OR=7). This difference is not statistically significant however is borderline (*p*-value= 0.088) (Table 1). Furthermore, sequence analysis revealed that all six patients had HCV genotype 3a (Table-2). The sequences obtained in this project had a 100% similarity to strain NCVI/PK1 from Pakistan (JN588558) (Figure 2).

These findings are in accord with results reported by other studies investigating the OCI status among the Iranian beta-thalassemia major patients, which indicated a prevalence rate of 3.3%-6.3% for OCI among the population (27, 32, 33). In this way, Bastani and colleagues found that six (5.7 %) 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 genotypes 1b, 3a, and 1a, respectively (27). In the same way, Kahyesh-Esfandiary et al., assessed the genomic HCV RNA in 48 PBMC specimens, collected from the Iranian patients with negative plasma HCV RNA; in that study, three (6.3%) of the subjects were also positive for OCI by RT-nested PCR, withal genotypes 1a and 3a were reported as the most common subtypes during HCV genotyping (33). Similarly, Ayadi and co-workers in another study from Iran showed that six (3.3%) of 181 beta-thalassemia cases had HCV RNA in their PBMC samples, and reported as OCI patients. Furthermore, HCV genotypes 1b (three subjects), 1a (two subjects), and 3a (one subjects) were identified in that study (32). As HCV genotyping results from the mentioned studies and the present work demonstrate, the HCV genotypes found among OCI patients are in line with the most

common HCV subtypes in Iran; 1a, followed by 3a and 1b (34). Additionally, previous investigations reported that the frequency of HCV infection among Iranian patients with beta-thalassemia major varies from 7% to 64% (33, 35), which is in agreement with the overt infection results from the present work so that 24 out of 91 (26.7%) subjects had HCV infection. On the other hand, it is well-known that HCV RNA may be positive in PBMCs of approximately 70 % of OCI subjects (36); thus, OCI may have a higher prevalence rate among the Iranian beta-thalassemia major patients, which need to be more evaluated during future studies by using liver biopsies as the gold standard for diagnosis of OCI (37). The current study found that 66.66% of the evaluated cases have seronegative OCI; hence, a negative result for anti-HCV Abs does not exclude HCV infection among beta-thalassemia patients. This study found a considerable prevalence rate of HCV infection (common infection and OCI) among the Iranian beta-thalassemia major patients. Regarding the significant mortality rate of HCV infection in thalassemic patients, OCI should be more considered 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 the prevalence rate of 6.7% OCI without evidence of the presence of HCV RNA in beta-thalassemia patients. Concerning serious complications and the clinical importance of OCI in beta-thalassemia patients, sensitive diagnostic methods for identifying HCV RNA in the PBMC should be implemented for all thalassemia patients when a liver biopsy is not available. Further studies with larger samples are needed to obtain a better estimate of OCI and to reveal the significance and repercussions of OCI in patients with beta-thalassemia major.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical Statement for Solid State Ionics – Diffusion and Reactions

I testify on behalf of all co-authors that our article submitted to Solid State Ionics – Diffusion and Reactions:

Title: Prevalence of Occult Hepatitis C virus infection in beta-Thalassemia major patients in Ahvaz, Iran

All authors: Sepideh Nasimzadeh, Azarakhsh Azaran, Shahram Jalilian, Manoochehr Makvandi, Seyed Saeid Seyedian, Bijan keikhaei, Fateme Jahangiri Mehr

1) This material has not been published in whole or in part elsewhere;
2) The manuscript is not currently being considered for publication in another journal;
3) All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

Date: 2020.9.11

Corresponding author's signature: Azarakhsh Azaran

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Figures

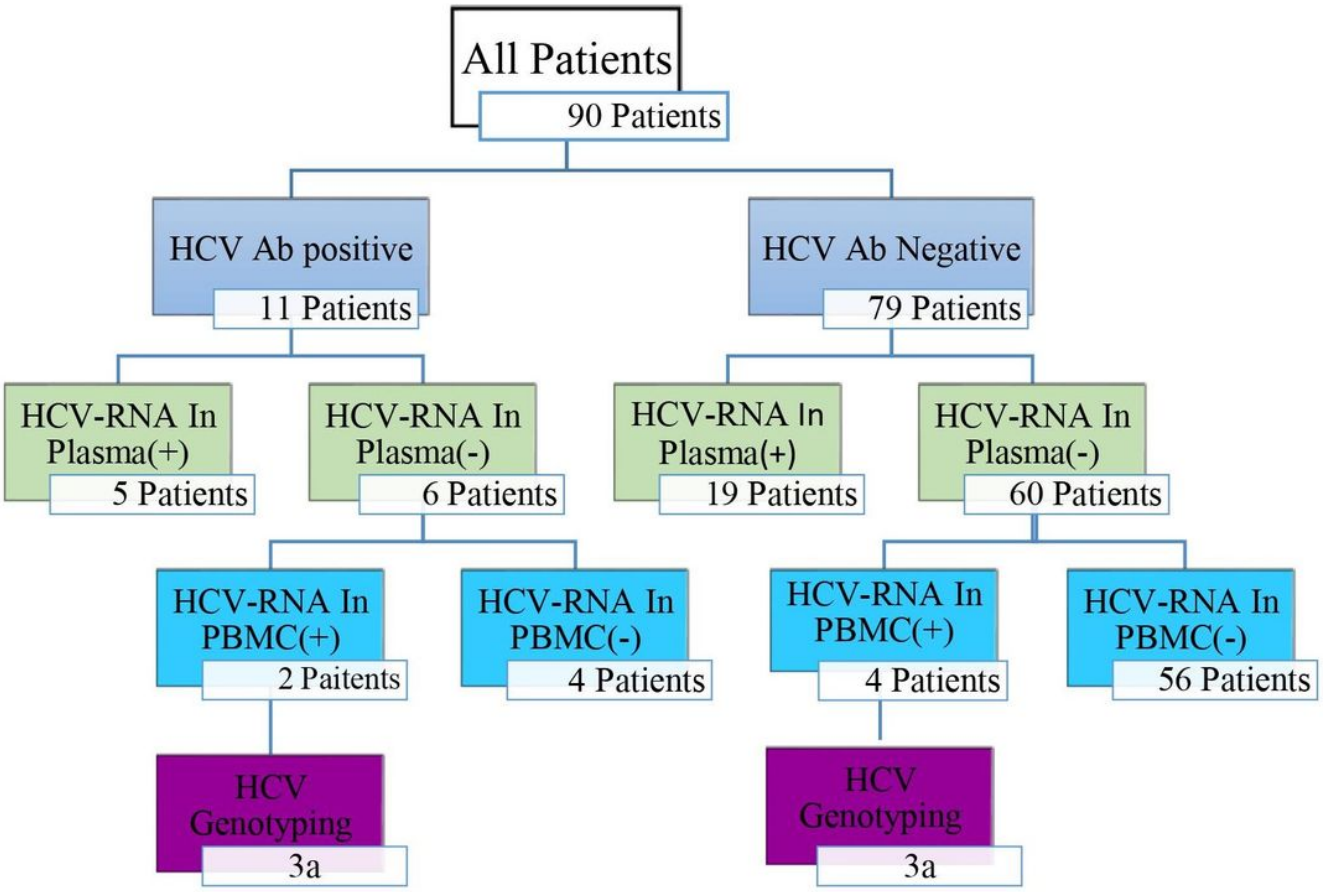

Figure 1

All the results obtained by serological and molecular experiments in this research.

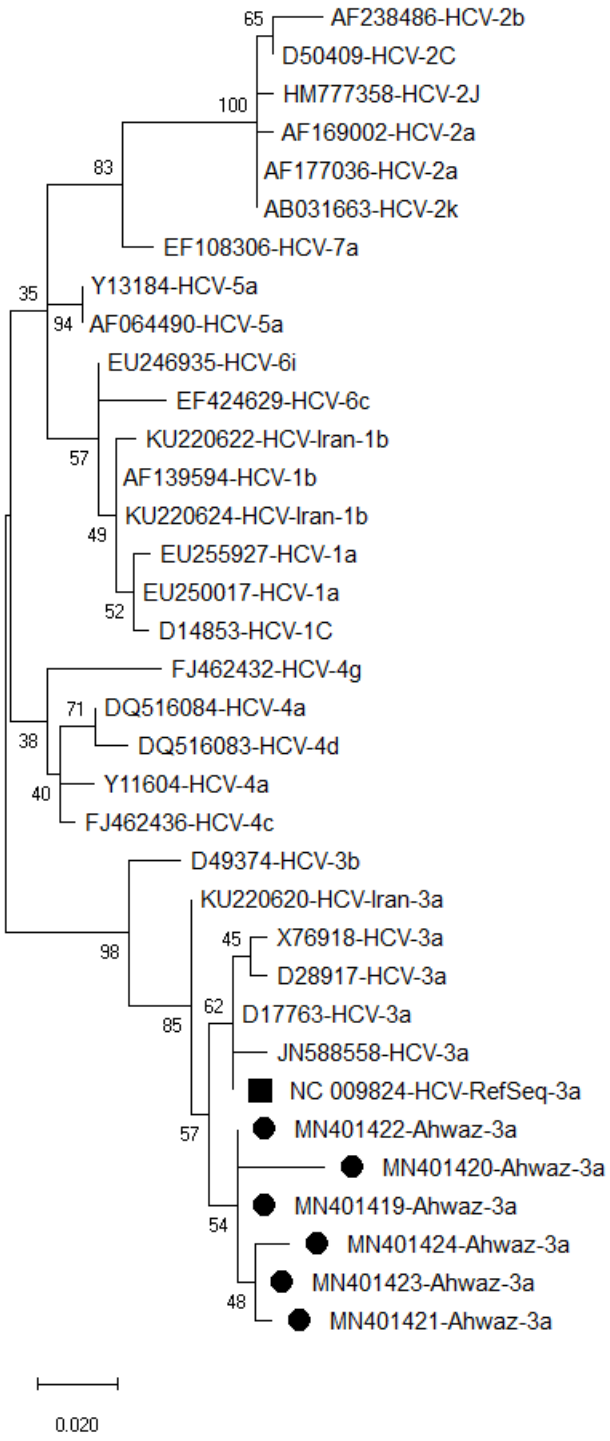

Figure 2

The phylogenetic tree based on the nucleotide sequences of HCV 5'-UTR region obtained from PBMC samples from participants who suffer from beta□thalassemia major with OCI. The tree was constructed using MEGA X based on maximum likelihood under the Tamura Nei-model. 1000 bootstrap replicates measured the tree's precision. In black circles the isolations obtained in this study are shown (□).In the black square, the reference sequence is shown.