

Determination of Genotype and Viral Load of HCV among Iranian Thalassemic Patients Suffering from Hepatitis

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ABSTRACT

AIM: Hepatitis C virus (HCV) is one of the most common causes of post-transfusion hepatitis in thalassemic patients. This study aimed to identify genotype distribution of HCV in patients with thalassemia in Iran due to the usefulness of HCV genotyping in viral transmission studies.

METHODS: We used a genotyping system based on nested RT-PCR of the core region with genotype-specific PCR primers for the determination of HCV genotypes followed by sequencing. Serum samples of 350 thalassemic patients (163 female and 187 male) suffering from hepatitis were used to extract the HCV genome. The Amplicon HCV Monitor test version 2.0 was used for viral load determination.

RESULTS: The frequencies of different genotypes were: 1a (20.5%), 1b (14.5%), 2a (12.5%), 2b (12.5%), 3a (24%), and 3b (16 %). Genotypes 1a and 3b were associated with a significantly ($p < 0.001$) higher viral load as compared to genotypes 1b and 2b. No statistical significance was observed for viral load among other HCV RNA genotypes.

CONCLUSIONS: The present study highlighted that the genotypes 1a and 3a are the predominant genotypes in Iran among thalassemic patients however the genotype 3a showed to be slightly more prevalent than 1a.

Key words: HCV; Genotyping; Viral load; RFLP; RT-PCR

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INTRODUCTION

Thalassemia is an autosomal recessive disease. The carrier rate ranges between 4 and 5.5% in different regions and racial groups^[1,2]. The management of thalassemia major is essentially comprised of

regular “safe blood transfusion” and a lifelong iron chelation therapy. Unfortunately, patients even those managed at relatively better management centers, are prone to develop two types of complications, i.e., those transmitted through blood transfusion (particularly hepatitis C) and the sequel of transfusion siderosis. Transfusion-transmitted diseases are the major challenge to the health of patients with hematologic disorders who need to receive regular blood products.

Improvement in screening of blood products during the 1990s has dramatically decreased the risk of infection via transfusion^[1]. The main routes of transmission in HCV are exposure to infected blood or blood product, intravenous drug use, infected medical equipment, tattooing, needle stick, hemodialysis, sexual activity, and organ transplantation^[2-6]. Infection with hepatitis C virus (HCV) has been identified as the major cause of post-transfusion non-A, non-B hepatitis^[3]. At present, hepatitis C virus (HCV) infection is the most prevalent transfusion-transmitted infection. Its seroprevalence in the general population is 0.5% to 1% but increases to 4% in cases of thalassemia in different settings^[4,5]. HCV infection in 60% to 80% of cases causes chronic hepatitis and imposes the risk of cirrhosis, end-stage liver disease, and hepatocellular carcinoma; therefore, its successful treatment has major value in the quality of life of patients infected with this viral disease. In many studies, associations between viral genotype, interferon responsiveness, the progression of disease, and the likelihood of developing hepatocellular carcinoma have been demonstrated^[6-14]. However, the basis for these associations is poorly understood, and the genotype distribution in multiply transfused patients with thalassemia is still unclear^[7]. HCV genotyping is being performed increasingly in clinical studies of HCV patients due to its usefulness in viral transmission studies. This study, which was performed on patients with thalassemia in Iran, aimed to identify genotype distribution of HCV in patients with thalassemia.

METHOD

Sample preparation

350 blood samples from patients with thalassemia were collected in 2014 (January until December) from Zafar Adult Thalassemia Clinic. The work has been approved by the Iran University of Medical Sciences (IUMS) ethical committee in compliance with the principles laid down in the Declaration of Helsinki. All patients gave informed consent to the work.

General patient data including gender, age, blood group, blood transfusion, and history of drug use were filed by their physicians (Table 1).

RNA extraction, cDNA synthesis

HCV RNA was extracted from serum samples of patients by using a RNX (extraction RNA kit) (RNX-Plus Solution CinnaGen CO Cat. No: RN7713C) according to the manufacturer’s instructions followed by conversion to cDNA. Briefly 5µg of the extracted RNA and 1 µl random hexamer (100 µM) were mixed and incubated at 65°C for

5 min and then chilled on ice followed by addition of 2 µl reverse transcriptase (200 U/ µl). The solution is filled with autoclaved double distilled water by volume 20 µl. The reaction mixture was incubated at 25°C for 5 min, followed by incubation at 60 for 50 min and 70°C for an additional 10 min and was then placed on ice. The cDNA was either used directly for nested PCR or stored at -20°C.

CR and genotyping primers

There is a high degree of sequence conservation found within the 5’ untranslated region (5’ UTR) of the genome, making this region a suitable target choice for most nucleic acid amplification-based detection assays.

For specific and nested PCR, four oligonucleotide primers from 5’-UTR of HCV were designed using Gene Runner primer design software (Hastings Software, Hastings, NY)^[8-9].

These primers were synthesized at the Cinna Gene Company (Tehran, Iran). The first round of PCR was done by external primers, the primer corresponding to HCV-1 sense oriented nucleotides -268 to -251 F1 (5’AGCGTCTAGCCATGGCGT 3’), and antisense nucleotides -4 to -22 R1 (5’GCACGGTCTACGAGACCT 3’). For the second round, the primer F2 (5’GTGGTCTGCGGAACCGG 3’) corresponded to sense-oriented nucleotides -199 to -183, and R2 (5’GGGCACTCGCAAGCACCC 3’) corresponded to antisense nucleotides -26 to -43. The PCR product was confirmed by Big Dye termination sequencing.

PCR Program

A volume of 10 µl PCR Master mix was mixed with 0.25 µl F1, 0.25 µl R1, 3µl cDNA, and mixed with DNase free water to a final volume of 25 µl and final concentration of 1µM for each primers. For the second round of nested PCR, 1 µl of the first round of PCR product was used with second pair of primers. The cycling program was as follows: The first round was carried out for 30 cycles which consisted of initial denaturation at 94°C for 5 min, denaturation at 94°C for 35 s, annealing at 58°C for 40 s, extension at 72°C for 45 s, and the final extension at 72°C for 5 min. The second round was followed for 25 cycles which consisted of initial denaturation at 94°C for 5 min, denaturation at 94°C for 35 s, annealing at 64°C for 40 s, extension at 72°C for 45 s, and the final extension at 72°C for 5 min. The 174-bp second PCR product was submitted to electrophoresis by using a 1.5% agarose gel in 0.5X TBE buffer and was visualized by ethidium bromide staining under ultraviolet light^[10].

Genotyping by RFLP

Total volumes of each nested-PCR product (25 µl) were divided into three tubes containing appropriate buffers. Restriction enzymes, *Apa* I, *Hinf* I, *EcoR* II, and *Bsh*1236 (Fermentas, Co.) were used as the following combinations: 1. *Apa* I / *Hinf* I; 2. *EcoR* II/*Hinf* I; 3. *Bsh*1236 I. The tubes were incubated with 1 U of the enzyme mixture for 3 h at 37°C. The digestion temperature was 37°C. If the samples could not be analyzed immediately after digestion, they were stored

Table 1 Patients’ data.

Group	All Patients	Female		Male			
	No. of Samples*	No. of Samples	ABO	Type of Thalassemia	No. of Samples	ABO	Type of Thalassemia
HCV & Thalassemia Positive	350	163	B+: 35 A+: 51 O+: 58 O-: 15 AB+: 4	Major: 133 Intermedia: 26 αthalassemia: 4	187	B+:53 B-:4 A+:54 A-:7 O+:50 O-:14 AB+:5	Major: 157 Intermedia: 26 αthalassemia: 4

*Blood transfusion 3 times a year.

at -20°C before analysis. The digested products were heated for 5 min and electrophoresed on 12% polyacrylamide gel. The genotypes were deduced from the fragmentation patterns of the digested PCR product^[11] (Table 2).

HCV Viral load

For viral load determination, the Amplicon HCV Monitor test version 2.0 (Roche Diagnostic Systems) was used according to the manufacturer’s instructions.

Statistical analysis

Statistical analysis was done with SPSS Statistics software, version 16. *P*-value <0.05 was considered as statically significant. Multiple/ Post Hoc Group Comparisons in ANOVA was used for viral load among HCV genotypes.

Table 2 Demonstrates cutting sites of Hinf I, Apa I, EcoR II, and Bsh1236 I restriction enzymes for different strains of HCV as published by Bukh *et al.* (25).

Genotype	Segment (bp)		
	Tube A (Apa I/Hinf I)	Tube B (EcoR II/Hinf I)	Tube C (Bsh1236 I)
1a	97	97	129
1b	97	97	99
2a	97	174	174
2b	174	174	174
3a	129	145	99
3b	97	145	99
4	97	145	129
5	97	174	99
6	97	97	174

RESULTS

Out of 350 total patients, 187 were male, and 163 were female. Patients were either recently infected by HCV or had previous positive PCR results showing history of HCV infection. The 5’- UTR of 350 HCV positive serum samples were amplified and digested by appropriate restriction enzymes for genotype determination. Figure 1 demonstrates the analytical polyacrylamide gel electrophoresis of HCV types 1a, 1b, 2a, 2b, 3a, and 3b after digestion of the amplified DNA with the selected restriction enzymes.



Figure 2 Schematic representation of the distribution of HCV positive thalassemic patients in different cities of Iran.

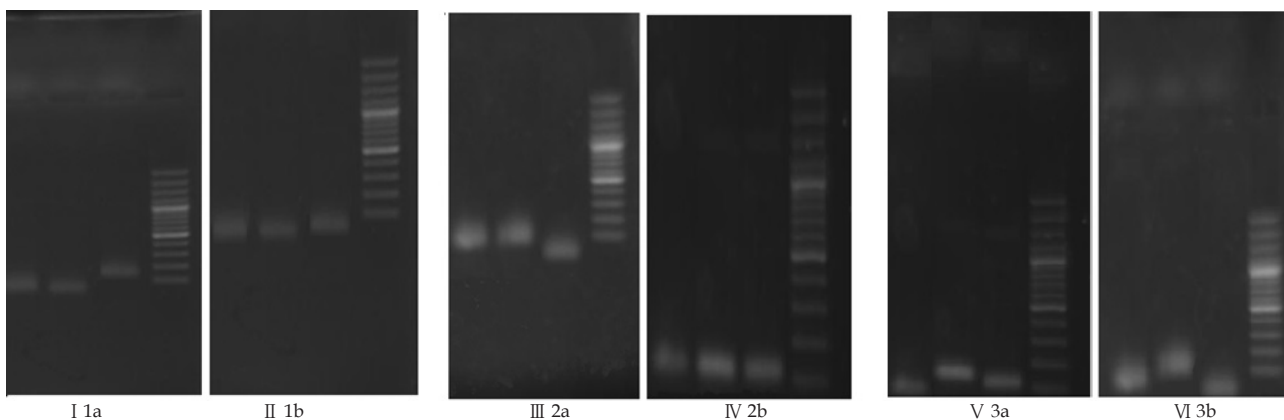


Figure 1 12% polyacrylamide gel electrophoresis of the digestion products of the amplified DNA from different genotypes. Marker; DNA 100 bp (lane M). I: Genotype 1a (Line 1:129, Line 2:97, Line 3:97 bp). II: Genotype 1b (Line 1:99, Line 2:97, Line 3:97 bp). III: Genotype 2a (Line 1:97, Line 2:174, Line 3:174 bp). IV: Genotype 2b (Line 1:174, Line 2:174, Line 3:174 bp). V: Genotype 3a (Line 1:129, Line 2:145, Line 3:99 bp). VI: Genotype 3b (Line 1:97, Line 2:145, Line 3:99 bp).

Table 3 HCV genotypes and loads in male and female patients.

Group	All Patients			Female			Male		
	No. of samples	Median age (year)	Median HCV load (IU/mL)	No. of samples	Median HCV load (IU/ml)	Median age (year)	No. of samples	Median HCV load (IU/mL)	Median age (year)
HCV positive	350	52	5194.58	163		55	187		48
Genotype 1a	72(20.5)	47	7323.9	30(18.2)	7200.9	50	42(22.4)	7412.4	45
Genotype 1b	49(14.5)	43	1223.9	21(12.9)	1221.9	48	28(15.8)	1269.7	43
Genotype 2a	44(12.5)	48	6472.9	26(16.1)	6475.6	47	18(9.3)	6469.9	39
Genotype 2b	45(12.5)	39	1005.9	25(15.1)	1013.9	39	20(10.2)	995.9	45
Genotype 3a	84(24)	39	5869.9	35(21.5)	6613.3	44	49(26.1)	5338.9	38
Genotype 3b	56(16)	45	7275.9	26(16.1)	7161.9	43	30(15.8)	7374.7	39

The genotyping results revealed HCV subtypes as following: 1a (20.5%), 1b (14.5%), 2a (12.5%), 2b (12.5%), 3a (24%), and 3b (16%).

In 72 patients with 1a subtype and 49 patients with 1b subtype, sequence analysis of 222 nucleotides in the NS-5b region clearly identified all of these isolates as subtype 1a and 1b respectively [data not shown]^[12].

Our results indicated that a high percentage of thalassemic patients infected with HCV in Iran were infected with 1a or 3a genotypes (Table 3).

DISCUSSION

HCV is highly variable, leading to the classification of at least six genotypes, each with several subtypes. The study of viral diversity provides a better understanding of the origin and dynamics of viral infections.

Epidemiological studies in different regions of the world showed that the HCV prevalence varies between different countries from 0.2 up to 40%. It is clearly seen that the incidence of HCV is higher among less developed nations. In Iran, HCV prevalence in the general population is less than 1%. Some cities showed a higher localization of HCV infected thalassemic patients, e.g. 46% Sari, 32% Tehran (Figure 2). In our study, HCV genotypes were; 1a (20.5%), 1b (14.5%), 2a (12.5%), 2b (12.5%), 3a (24%), and 3b (16%).

It has been reported that genotype 1b in Turkey, genotypes 3a and 3b in Pakistan, 1a, 1b, 2a, 2k and 3a in Uzbekistan, and 1g in Lebanon are dominant genotypes^[13].

Genotype information is important when HCV treatment is being considered, since some genotypes respond more favorably to medications. Genotype also determines the length of therapy. For example, Peg Interferon therapy treatment for genotypes 2 and 3 requires only 24 weeks while genotypes 1 and 4 require 48 weeks. In addition to treatment purposes, detection of HCV genotypes in different regions can be used for the purpose of molecular epidemiology^[14].

Investigators of HCV genotyping have used sequence analysis of HCV NS5, Core, E1 and 5'-UTRs. However, direct sequencing is not practical on a large scale. RFLP has been used widely for this aim, especially in the screening of a large number of specimens^[11].

In this study, the viral loads of HCV were analyzed among thalassemic patients who were HCV positive. It was revealed that the detected HCV genotypes are independent factor in association with the measured viral loads. Although similar analyses have been reported for patients infected with genotype 6 comparing with those infected with genotypes 1, 2, and 3, no statistical differences were shown^[10,14]. However, in this study, Genotypes 1a and 3b were associated with a significantly ($p < 0.001$) higher viral load as compared to genotypes 1b and 2b. No statistical significance was observed for viral load among other HCV RNA genotypes (Table 3). To conclude, the present study highlighted that the genotypes 1a and 3a are the predominant genotypes in this geographical region among thalassemic patients however the genotype 3a showed to be slightly more prevalent than 1a.

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