

## Original Research Article

Molecular Epidemiology of Hepatitis C Virus Genotypes in Patients with  
Thalassemia Major in Golestan Province, IranSareh Zhand<sup>1</sup>, Masuod Bazuori<sup>1</sup>, Alijan Tabarraei<sup>1</sup>, \*Abdolvahab Moradi<sup>1</sup><sup>1</sup>Department of Microbiology, School of Medicine, Golestan University of Medical Sciences, Gorgan, Iran**ABSTRACT**

**Introduction:** In Iran, hepatitis C virus (HCV) is the most prevalent cause of chronic hepatitis and cirrhosis in patients with hemophilia, thalassemia, and renal failure. Recent studies suggest that patients infected with different HCV genotypes have different clinical profiles, severity of liver disease and response to therapy. Several molecular methods targeting different HCV genomic regions have been introduced for genotyping. Direct sequencing of amplified PCR products is the gold standard method, followed by phylogenetic analysis of clinical material. The aim of this study was to determine genotypes of HCV-infected patients with thalassemia in Golestan Province, Iran.

**Materials and Methods:** This cross-sectional study included 217 patients (mean age:  $21.82 \pm 16$  years, 50.7% male) with thalassemia major. Enzyme-linked immunosorbent assay (ELISA) was used for detection of HCV antibodies. Positive HCV-Ab samples were confirmed by reverse transcription polymerase chain reaction (RT-PCR) and sequencing. HCV genotypes were determined by aligning nucleotide sequences of patients with the standard nucleotide sequences obtained from GenBank (accession number: AB520610). **Results:** Of 217 patients with thalassemia major, 14 (6.45%) were found as anti-HCV-positive in the ELISA test. Among them, two patients (14.28%) had positive RT-PCR results. In addition, all patients were infected with HCV genotype 1a. **Conclusions:** Genotype 1a is the predominant HCV genotype in patients with thalassemia major in the Golestan province, Iran.

**KEYWORDS:** Hepatitis C virus, Genotype, Thalassemia major, Golestan Province

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**INTRODUCTION**

Hepatitis C virus (HCV) is a major health problem affecting 170 million people worldwide. According to world health organization, the seroprevalence of HCV is about 1% in Western countries and North America, and 3-4% in some Mediterranean and Asian countries [1]. In Iran, the infection is emerging mostly due to increasing rates of intravenous drug abuse and needle sharing [2]. In addition, increase in number of centers providing hemodialysis and transfusion facilities for patients with hemoglobinopathies generated new sources of susceptible populations in Iran [3]. HCV infection is the leading cause of chronic hepatitis worldwide, progressing to liver cirrhosis in approximately 20% of patients after 10 years and to hepatocellular

carcinoma (HCC) in a subset of patients with a 3% annual incidence rate [4, 5]. Chronic hepatitis C is often silent, and usually diagnosed by routine serological, biochemical and radiological tests. Many attempts have been made to identify the natural history and progression of hepatitis C infection, but several aspects remain to be elucidated. The rate of disease progression is variable and depends on several factors such as age at infection, gender, genotype/subtype, viral load, and mode of infection. In Iran, HCV infection is the most prevalent cause of chronic hepatitis and cirrhosis in patients with hemophilia [6], thalassemia [1] and renal failure [7]. Accumulation of nucleotide substitution in the HCV genome results in diversity and

evolution into different genotypes, subtypes and quasispecies [8, 9]. Six major genotypes (HCV-1 to HCV-6) have been described so far, each containing multiple subtypes (e.g., 1a, 1b, etc.). Each of these genotypes is equally divergent from one another and varies by as much as 35% of nucleic acid content, while subtypes within a typical genotype differs from each other by 20-23% [10, 11]. There is increasing evidence suggesting that patients infected with different HCV genotypes have different clinical profiles, severity of liver disease and response to alpha-interferon therapy [12, 13]. Patients infected with HCV genotypes 1 and 4 achieve sustained remission following a 48-week course of treatment, while a 24-week course of therapy is sufficient for genotypes 2 and 3 [14]. Genotypes 1 and 4 show more resistance to pegylated interferon plus ribavirin therapy compared to genotypes 2 and 3. Therefore, different HCV genotypes require different duration and dosage of antiviral therapy [15, 16]. Furthermore, results of HCV genotyping can help determine mode of infection transmission and molecular epidemiology [17]. Although there are several methods of HCV genotyping, the gold standard method is direct sequencing of amplified PCR products. Given that approximately 1% of Iranian population is infected with HCV, chronic hepatitis C remains a serious health problem with considerable burden on the healthcare system [2].

Geographical distribution of subtypes differs significantly. In North America and Northern Europe, subtype 1a is the most common, followed by 2b and 3a [18,19]. In Southern and Eastern Europe, the most common subtype is 1b, followed by genotypes 2 and 3 [20, 21]. In other parts of the world, genotypes other than 1, 2 and 3 are more common. Genotype 4 seems to be confined to the Middle East [22] and Central Africa [23, 24], while genotype 5 has been

isolated almost exclusively from South Africa where it predominates, followed by genotypes 1, 2, 3 and 4 [25]. Genotype 6 has been reported from Hong Kong, Vietnam and throughout Southeast Asia [12, 26]. It is thought that genotyping pattern of Iranian patients is similar to European and North American patients since all reports confirm that genotypes 1 and 3 are the most distributed in Iran [27-29]. Previous studies have shown that genotypes 1a and 3a are the most common genotypes in Iran [27, 28, 30, 31], which is different from the dominant genotypes of other Middle Eastern countries [30, 32, 33]. This study aimed to determine the genotypes of HCV in patients with thalassemia in the Golestan Province, Iran.

## MATERIALS AND METHODS

### *Clinical samples*

This cross-sectional study was conducted on 217 patients with thalassemia major referred to Thalassemia Center at Taleghani Hospital in Gorgan, Iran. Analyses included history taking, physical examination, and periodic clinical and serological evaluations. Plasma samples obtained from all patients were stored at -80 °C until genotyping assay. The research project received approval from the local ethics committee (code of practice: 831/35/p/g, June 2013).

### *HCV antibody detection by enzyme-linked immunosorbent assay (ELISA)*

The diagnosis of hepatitis C infection was made based on detection of anti-HCV antibodies in plasma using third generation commercially available ELISA kits (GmbH, Germany). Positive samples in the ELISA test were confirmed with RT-PCR for increasing the sensitivity.

### *RNA extraction and cDNA synthesis*

Viral RNA was extracted from anti-HCV-positive samples using High Pure Viral Nucleic acid kit (Roche, Germany) according to the manufacturer's protocol.

Briefly, 200  $\mu$ l of plasma were treated with 200  $\mu$ l of guanidine hydrochloride supplemented with poly (A) carrier RNA, and 50  $\mu$ l of proteinase K. After vortexing and heating the samples to 72 °C for 10 minutes, nucleic acids specifically bound to the surface of glass fibers precast into a column. Non-specifically bound material was removed by washing solution provided in the kit. Finally, nucleic acids were eluted in 50  $\mu$ l of elution buffer. Then, 0.5 ng of the eluted RNA was used to carry out reverse transcription. Next, cDNA synthesis was done using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Germany) using random hexamers. Five  $\mu$ l of the cDNA synthesized were used for the PCR reaction.

**Table 1: Oligonucleotide primers used for amplification of HCV core region [34].**

Primer	Position	Direction	Oligonucleic sequence (5→3)
HCV-1	139-158	Sense	CGCGCGACTAGGAAGACTTC
HCV-2	410-391	Antisense	ATGTACCCCATGAGGTCGGC

All reactions were performed using positive and negative controls. After DNA amplification, the presence or absence of bands specific to 271 bp amplified DNA was evaluated on 2% agarose gel stained with DNA safe Stain (Cinnagen). After amplification, the positive PCR products were subjected to automated sequencing (Macrogen Inc., Korea). HCV genotyping/sub-typing was performed by aligning the nucleotide sequences from each sample with the standard hepatitis C genotype 1a sequence (Accession number: AB520610) from the GenBank database.

## RESULTS

### *Prevalence of HCV among thalassemia patients*

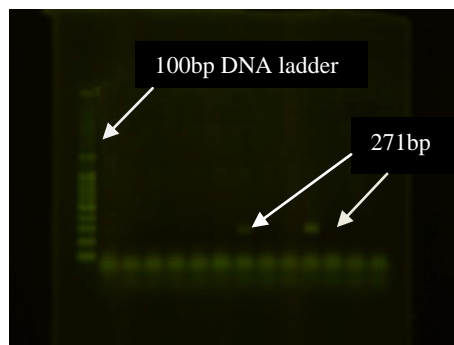
Among 217 thalassemia patients studied, 50.7% were male and 49.3% were female. The mean age of patients was 21.82±16 years with an age range of 15-57 years. Serological data showed that 14 (6.45%)

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### *HCV genotyping assay*

HCV genotype was determined by type-specific primers and sequencing. After cDNA synthesis, nucleic acid amplification was carried out. The reaction solution contained 0.4  $\mu$ l of HCV-1 and HCV-2 primers (sense and anti-sense primers for the core region, respectively, Table 1), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, and 2.5 units of Taq DNA polymerase (Genet Bio, Korea). Thermocycler conditions were as follows: initial denaturation at 94 °C for 3 min, preliminary 30 cycles of amplification in three steps: 94 °C for 1 min, 57.5 °C for 1 min, 72 °C for 1 min, followed by final extension at 72 °C for 2 min.

Patients were anti-HCV positive in the ELISA test, 2 (14.28%) of which had positive RT-PCR results for core region of HCV for genotyping. The prevalence of identified genotypes was determined for four age groups. The highest rate was observed in patients aged 40-41 years.



**Figure 1: Electrophoresis pattern of the 271 bp PCR product amplified from core region of the HCV in anti-HCV positive thalassemia patient. Column 1: 100bp DNA ladder, columns 8 and 11: positive PCR Products, column 9: negative PCR products**

### Results of HCV genotyping

Alignments of the nucleotide sequence from patients with the standard nucleotide

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Query_ 529 AGCCTATCCCCAAGGCGCGTCGGCCCCGAGGGCAGGACCTGGGCTCAGCCCGGGTACCCTT 588
          |||
Sbjct_ 27  AGCCTATCCCCAAGGCGCGTCGGCCCCGAGGGCAGGACCTGGGCTCAGCCTGGGTACCCTT 86

Query_ 589 GGCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCCCCCGTGGCT 648
          |||
Sbjct_ 87  GGCCCTCTATGGCAACGAGGGCTGCGGGTGGGCGGGATGGCTCCTGTCCCCCGTGGCT 146

Query_ 649 CTCGGCTAGCTGGGGCCCCACAGACCCCGGCGCAGGTCACGCAATTTGGGTAAGGTCA 708
          |||
Sbjct_ 147 CTCGGCTAGCTGGGGCCCCACAGACCCCGGCGTAGGTCGCGCAATTTGGGTAAGGTCA 206

Query_ 709 TCGATACCCTCACGTGTGGCTTCGCCGACCTCATGGGGTACA 750
          |||
Sbjct_ 207 TCGACACCCTCACGTGCGGCTTCGCCGACCTCATGGGGTACA 248
  
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Figure 2: Alignment of positive PCR products with the reference sequence with 98.8% similarity

### DISCUSSION

HCV is responsible for up to 56% of all diagnosed cases of viral hepatitis in the local community [35, 36]. According to epidemiological studies, this virus is distributed worldwide with prevalence varying from 0.2% up to 40% in different countries. It is well demonstrated that the incidence of HCV is higher in less developed countries. The prevalence of HCV in Iran is less than 1% [37], similar to blood donors in Northern European countries [38]. Higher rates have been reported in Egypt (14.5%) and Southeast Asian countries such as India (1.5%), Malaysia (2.3%) and the Philippines (2.3%) [17]. Genotypes 1b in Turkey, 3a and 3b in Pakistan, 1a, 1b, 2a, 2k and 3a in Uzbekistan, and 1a in Lebanon have been reported as the dominant genotypes [39]. Genotype 4 is the main genotype circulating in most Arab countries. Genotype 4a in Bahrain and 4 in Saudi Arabia were detected in 50% of patients, while genotype 1b was found in nearly 40% of patients [40]. Zali et al. studied the prevalence of HCV genotypes in Tehran and reported subtype 1a as the predominant genotype (42%), followed by subtype 3a (35%) [27]. Study of Samimi-

sequence showed that all samples have been infected with HCV genotype 1a.

Rad et al. on anti-HCV antibody-positive patients from Tehran and five other cities showed that genotype 1a (47%) is predominant, followed by genotypes 3a (36%), 1b (8%) and 4 (7%) [28]. Ahmadipour et al. found genotypes 1a (52.88%), 3a (27.57%) and 1b (14%) in Iranian HCV-infected patients using the PCR-RFLP method [3, 28]. Geographical differences may help predict the origin of HCV [41]. Based on our findings, All HCV-infected patients with thalassemia major in the Golestan Province are infected with genotype 1a. Similar to our study, study of Mirmomen et al. on 732 patients with  $\beta$ -thalassemia major/intermedia from provinces of Tehran, Kerman, Qazvin, Semnan and Zanjan showed that 19.3% of the patients had positive HCV antibody test results [42]. Previous single-center studies on Iranians with  $\beta$ -thalassemia reported the prevalence of HCV infection between 16-64% [2]. The present study found that 6.45% of patients with  $\beta$ -thalassemia were HCV-infected. Inconsistent with our study, a study in Pakistan on 75 patients with thalassemia major reported the prevalence of HCV infection as 42%, using third

generation ELISA kits [43]. Our findings are similar to the study of Bhavsar et al. that found 18% of patients with thalassemia major have been infected with HCV [44]. Our findings are also consistent with findings of Wonke et al. (11.1%) [45] and Chaudhary et al. in India, but inconsistent with the results of Shah et al. in Pakistan (56.8%) [46] and Wanachiwanawin et al. in Thailand (20.2%) [47]. The discrepancy in results may be due to the different methods used for detecting anti-HCV antibodies. The prevalence of anti-HCV antibodies in thalassemia patients from different regions varies between 11 and 75% [45, 48, 49]. Another study by Oza et al. on 193 children with thalassemia major reported the prevalence of HCV infection as 7.8% [50]. In a systematic review by Alavian et al. in Iran [51], of 5229 patients with thalassemia, 941 (17.9%) were anti-HCV antibody-positive. Positive ELISA results were confirmed by the RIBA test in 568 cases, and the highest and lowest prevalence rates were seen in Semnan (32%) and Zanjan (2%), respectively. The rate of HCV infection in thalassemia patients from Pakistan, Saudi Arabia and Egypt was 45, 63 and 69%, respectively [51]. Lower prevalence rate in Iran compared to these countries could be due to the screening systems for blood transfusion in Iran. Results of the present study are also similar to findings of Tamaddoni et al. that showed 10.6% of patients with  $\beta$ -thalassemia major were positive for anti-HCV antibody [52]. Study of Langarodi on 206 thalassemia patients found the overall prevalence rate of anti-HCV antibodies as 15%, while 14.8% of these anti-HCV antibody-positive patients were RIBA-II positive [53]. Study of Ataei et al. in Iran, reported the prevalence of HCV as 8% among 466 patients with thalassemia major [54], which is similar to our findings. In 2010, a study reported the seroprevalence rate of 0.5% among healthy

Iranian blood donors [35].

Genotype 1a is most commonly found in Northern Europe and North America [55, 56]. It was also found as the most prevalent genotype in our study, which is consistent with some other studies in Iran [57-59]. However, study of Hosseini-Moghaddam et al. on 45 hemodialysis centers in Tehran, revealed that genotypes 3a (30.3%) and 1a (28.8%) were the most prevalent genotypes, followed by genotypes 1b (18.2%), 4 (16.7%) and 3b (3%) [60]. Study of Somi et al. in East Azerbaijan reported the frequency of genotypes 1a, 3a and 1b as 74.6, 5.5 and 5.5%, respectively [30]. In study of Keyvani et al., unlike other hemodialysis centers where subtype 1a was the most prevalent genotype, the prevalence of genotype 3a in hemodialysis centers of Rasht was higher than that of 1a [59]. Genotype 3a is also prevalent in countries such as Pakistan and India [26].

Similar to studies of Somi et al. [30] and Kabir et al. [55], our study showed no significant difference in genotype distribution in terms of age and gender. HCV genotyping plays an important role in the clinico-epidemiological manifestations of HCV infection, its efficient clinical management and prognosis in chronic cases, and ultimately in vaccine development [61]. Several studies have been conducted in Iran on the distribution of HCV genotypes among high-risk populations [62]. Different factors such as geographic location as well as mode and year of transmission affect the distribution of HCV genotypes [63]. Therefore, studying the distribution of HCV genotypes in different countries can help clarify the epidemiology and evolution of HCV, and act as a useful tool for identifying high-risk groups and different routes of transmission [64]. The prevalence of HCV genotypes in different parts of Iran have been investigated using different methods [61, 65]. In study of Mousavi et al. on 509

anti-HCV antibody-positive patients in Hormozgan, 238 (46.7%) patients were intravenous drug users. In addition, 316 (62.1%), 117 (23%) and 76 (14.9%) patients had genotypes 1a, 1b, and 3a, respectively [66]. According to most studies, genotype 1 is the predominant HCV genotype in Iran, followed by genotype 3 [61, 65, 67]. Inconsistent with our findings, study of Hajia et al. reported genotype 3a as the predominant HCV genotype (46.6%), followed by genotype 1 (43.2%) [68]. Correlation of HCV genotypes with demographic and epidemiological variables show that genotype 1a is evenly distributed all over the country in both genders and have affected all age groups with different ethnicities, indicating that the genotype has been present and circulated in our community for a long time [58].

## CONCLUSIONS

Our study shows that genotype 1a is the dominant HCV genotype in patients with thalassemia major in the Golestan province, Iran. There are more than 25,000 patients with  $\beta$ -thalassemia major in Iran. Among these patients, there might be around 5,000 HCV-infected patients at risk of liver failure and hepatocellular carcinoma. Further investigations with larger study population are necessary to determine the major genotypes that cause HCV infection in patients with various clinical conditions.

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