Genetic diversity of HCV among various high risk populations (IDAs, thalassemia, hemophilia, HD patients) in Iran

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1. Introduction

With more than 3% of the world’s population infected by hepatitis C virus (HCV), this disease has become a major public health challenge. HCV infection is a severe health problem, causing approximately 20% of acute hepatitis, 80% of chronic hepatitis, 40% of hepatocellular carcinoma, and 30% of liver transplantations. HCV infection is endemic in Iran and the prevalence rate varied from 0.08% to 1.3% in the central and northern of Iran, respectively. Some groups of patients in different exposure categories such as individuals with haemophilia, thalassemia, end patients with stage renal disease under hemodialysis (HD), and intravenous drug addicts (IDAs) are at high risk of acquiring HCV infection.

HCV is a small, enveloped, single-stranded, positive sense RNA virus with a large genetic heterogeneity rate. Based on a nucleotide sequence divergence, isolates have been classified into 6 major genotypes and more than 70 subtypes. These genotypes differ by 31% to 34% in their nucleotide sequence and by around 30% in their amino acid sequence. The prevalence of HCV genotypes differs substantially in distinct geographical regions. On the other hand, HCV genotypes 1, 2, and 3 distribute all over the world with some variation in their prevalence from one geographic region to others. HCV genotyping is mainly useful for the clinical management of infected patients and for facilitating decisions on therapy, as genotypes 1 and 4 are less likely than genotypes 2 and 3 to respond to interferon.

HCV is principally acquired and transmitted by parenteral route including blood transfusion, intravenous drug abuse...
and nosocomial transmission. The high-risk groups of HCV infection include hemophiliacs, intravenous drug addicts, and patients with end-stage renal disease under hemodialysis[7]. There is no vaccine against HCV and available treatments are long, difficult, and expensive and will not work for all the patients. In Iran, HCV infection, mostly occurred due to problems like increasing population of drug addicts and common needle-sharing among, using blood or it’s infected derivatives in surgery or to survival of thalassemia, hemophilia and patients under hemodialysis[7]. Although frequent blood transfusion led to continue survival of these specific patient groups, but due to non-compliance with important health tips on infection control in dialysis units and other health care centers, these patients are at high risk of affecting blood borne pathogens, particularly HCV[9,10]. Precise data of HCV genotypes pattern and continuous monitoring of the genetic diversity of HCV isolates, especially among high-risk individuals in our community, is essential for understanding of epidemiology, pathogenesis, and successful management of the patients.

Since there is inadequate data about genotype pattern of HCV infection among high risk groups from different regions of Iran especially for north of Iran, we carried out this study to provide molecular epidemiology data of HCV genotype pattern among high-risk individuals.

2. Materials and methods

2.1. Study population

A cross-sectional study was carried in Mazandaran province, north of Iran, during July 2009 to September 2011. Individuals with HCV infection who were recruited by the Blood Bank of Sari, Department of Infectious Diseases, Razi Hospital, Thalassemia and Hemophilia Associations, and Center for Prevention and Treatment of Chemical Dependency. A questionnaire was used in face to face interview to collect some demographic information, epidemiological features such as; age, sex, history of intravenous drug addiction, tattooing, treatment with interferon, duration of HCV infection, and history of hospitalization. All thalassemia patients were beta thalassemia major and received regular blood transfusions at 4 week intervals to maintain haemoglobin level at 10–13 g/dL, along with regular therapy with deferoxamine. All hemophilia patients were hemophilia A and they have mainly injected whole blood cells or blood derivatives such as clotting factor concentrates, cryoprecipitate and fresh frozen plasma. Most of the IDAs population studied was multiple drug users with high levels of consumption of heroine (37.2%), crack (44.2%), cocaine (6.7%), opium paste (51.6%). The duration of drug use varied from 2 to 10 years. The protocol used in the present study was approved by the Ethical Committee of Mazandaran University of Medical Sciences and written informed consent was obtained from each patient.

2.2. Serological test

Plasma samples were obtained from four high risk patient groups chronically infected with HCV, thalassemia, hemophilia, end stage renal disease under HD, IDAs, all of whom were seronegative for hepatitis B virus, human immunodeficiency virus, and autoimmune markers. The presence of anti–HCV antibody was tested by a third-generation enzyme immunoassay (Ortho–Clinical Diagnostics, Raritan, NJ, USA) detecting reactivity to three HCV recombinant proteins (c22–3, c200 and NS5) originating from four regions of viral genomes: core, NS3, NS4 and NS5. All samples reactive on ORTHO HCV Version 3.0 ELISA Test System were tested with the confirmatory recombinant immunoblotting assay, RIBA (Chiron RIBA HCV 3.0 SIA; Chiron Corp., Emeryville, CA, USA) according to the manufacturers’ instructions.

2.3. HCV–RNA detection and genotyping

All HCV positive plasma samples were submitted to RNA extraction by using QIAamp® Viral RNA Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer’s instructions. For complementary DNA (cDNA) synthesis, a transcriptase reaction was performed using MMLV–reverse transcriptase, random hexamer, dNTP, and RNAsin inhibitor in the final volume reaction 50 μL according to the following thermal profile: 70 °C for 10 minutes, 25 °C for 15 minutes, 37 °C for 60 minutes and 95 °C for 15 minutes in a thermocycler (Eppendorf Mastercycler, Eppendorf, Hamburg, Germany). HCV genotypes were determined on all HCV–RNA positive samples by using AmpliSens® HCV–genotype kit (Russia, Moscow) according to manufacturer’s instructions. The Kit can specify genotype and subtypes 1a, 1b, 2, 3a, and 4 with different product sizes 388, 395, 412, 286 and 227 base pairs. The hot-start protocol was applied to decrease the level of non–specific priming. Thermal profile of PCR program was as follow: initial denaturation in 95 °C for 5 min follow by 35 cycles of 94 °C for 1 min, 68.5 °C for 1 min, 72 °C for 1 min, and final extension in 72 °C for 1 min. PCR products were electrophoresed on 2.5% agarose gel and visualized by staining with ethidium bromide under ultraviolet light. To avoid cross–contamination between samples, standard precautions were used in all manipulations. Separate areas were used when handling reagents and samples and manipulating amplified products.

2.4. Statistical analysis

Clinical and demographic continuous data were summarized as mean±SD, and categorical data were summarized by absolute frequencies or percentages. Comparisons of continuous or categorical variables were preformed with ANOVA or chi square tests. All P values were evaluated in a two–sided model, and P<0.05 was considered statistically significant. All analysis performed
3. Results

3.1. Demographic and risk factors among study population

Among the 187 positive anti-HCV samples, only 135 (72.2%) gave HCV-specific positive signals by PCR of the 5'–UTR of HCV. The group of 135 Iranian was composed mainly of males 94 (69.6%). The mean age was (35.1 ± 12.0) years. Of the participants, 34 (25.18%) thalassemia, 31 (22.96%) HD, 33 (24.45%) hemophilia and 37 (27.41%) IDAs were found to be positive for anti-HCV and HCV-RNA. The baseline characteristics of the study population were shown in Table 1. The distribution of age and gender in the study subgroups were statistically significant differences. On the other hand, thalassemic individuals and HD patients had the minimum and maximum mean age, respectively (P < 0.0001). Meanwhile, hemophilia and IDAs were prominently males (P < 0.0001). Seventy six (56.9%) out of 135 individuals had hospitalization history and thalassemia had more times hospitalization than the other groups. Forty eight percent of patients reported a history of surgery for at least one time. A total of 20 (58.8%) out of 34 thalassemia patients were splenectomized. Twenty four out of 135 patients had a history of tattoo, among them; IDAs were prominent (64.9%). Fifty two (38.5%) HCV-RNA individuals underwent pegylated interferon alpha therapy. Thalassemia and hemophilia received this treatment more than the other groups. The mean levels of liver enzymes such as AST and ALT were found to be approximately more than 1.5 times higher than their normal levels and were found to be statistically significant between HCV–RNA infected thalassemia, IDAs, and HD patients. On the other hand, the mean levels of ALT in thalassemia and hemophilia was found to be two times higher than their normal levels (<40 IU/L).

3.2. HCV–RNA prevalence and HCV genotypes

Genotype analysis was performed on the HCV RNA–positive samples and Table 2 summarizes HCV genotypes in our participants. Over all, the most identified HCV type was genotype 3a (51.1%) followed by 1a (27.4%), 1b (8.2%). On the other hand, 16 (11.86%) out of 135 HCV RNA–positive participants were infected with more than one genotype or subtypes as follow; 1a/1b in 11 (8.2%), 2/3a in 3 (2.2%), and 1a/1b/3a in 2 (1.5%). In addition, we could not type 2 (1.5%) of our patients by using Amplisens HCV genotyping kit. Stratification of our participants to four high risk groups revealed that HCV subtype 3a was more prominent in the high risk groups except IDAs. So that the prevalence of subtype 3a were 24 (77.42%), 15 (44.12%), and 25 (75.76%) in HD, thalassemia, and hemophilic patients, respectively. The prevalence of type 1 in IDA subjects was higher than that type 3a (56.76 vs. 13.51, respectively). Interestingly, among all study groups, subtype 1b and mixed subtypes 1a/1b were only prominent in IDAs participants (27.03%, 29.73%, respectively). Mixed types of 2/3a were only found in 3 (8.82) thalassemic patients. Analysis of HCV genotype frequency across thalassemic patients with positive history of surgery revealed that only subtype 1a found in splenectomized patients.

### Table 1
Basic characteristics of different HCV RNA positive high risk patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total (n =135)</th>
<th>Thalassemia (n =34)</th>
<th>Hemophilia (n =33)</th>
<th>HD (n =31)</th>
<th>IDAs (n =37)</th>
<th>P–value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>35.1±12.0</td>
<td>26.6±4.9</td>
<td>32.4±8.7</td>
<td>47.4±13.1</td>
<td>34.9±9.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>94/41</td>
<td>17/17</td>
<td>31/2</td>
<td>13/18</td>
<td>33/4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Duration of infection (month)</td>
<td>60.8±57.7</td>
<td>71.8±42.4</td>
<td>62.1±46.7</td>
<td>90.6±40.7</td>
<td>22.0±11.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hospitalization</td>
<td>76 (56.9)</td>
<td>27 (79.4)</td>
<td>8 (24.2)</td>
<td>19 (51.4)</td>
<td>22 (59.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>History of surgery</td>
<td>48 (35.6)</td>
<td>20 (58.8)</td>
<td>5 (15.15)</td>
<td>4 (12.9)</td>
<td>19 (51.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tattoo</td>
<td>24 (17.8)</td>
<td>–</td>
<td>1 (3.0)</td>
<td>–</td>
<td>23 (64.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Interferon therapy</td>
<td>52 (38.5)</td>
<td>19 (55.9)</td>
<td>15 (45.5)</td>
<td>10 (32.3)</td>
<td>8 (21.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>64.5±24.7</td>
<td>75.6±32.3</td>
<td>61.2±30.8</td>
<td>30.92±15.3</td>
<td>69.8±44.3</td>
<td>0.015</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>70.5±43.8</td>
<td>81.9±42.9</td>
<td>58.8±25.2</td>
<td>28.45±17.7</td>
<td>80.1±45.2</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Normal levels: AST, <40 IU/L; ALT, <40 IU/L.

### Table 2
Distribution of HCV genotypes among different high risk group of patients.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total (n [%])</th>
<th>HD (n =31)</th>
<th>Thalassemia (n =34)</th>
<th>Hemophilia (n =33)</th>
<th>IDAs (n =37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>37 (27.40)</td>
<td>6 (19.36)</td>
<td>13 (38.24)</td>
<td>7 (21.21)</td>
<td>11 (29.73)</td>
</tr>
<tr>
<td>1b</td>
<td>11 (8.20)</td>
<td>0 (0.00)</td>
<td>1 (2.94)</td>
<td>0 (0.00)</td>
<td>10 (27.03)</td>
</tr>
<tr>
<td>3a</td>
<td>69 (51.10)</td>
<td>24 (77.42)</td>
<td>15 (44.12)</td>
<td>25 (75.76)</td>
<td>5 (13.51)</td>
</tr>
<tr>
<td>Mixed genotype</td>
<td>1a, 1b</td>
<td>11 (8.20)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>2, 3a</td>
<td>3 (2.20)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>1a, 1b, 3a</td>
<td>2 (1.50)</td>
<td>1 (3.22)</td>
<td>1 (2.94)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Not typable</td>
<td>2 (1.50)</td>
<td>0 (0.00)</td>
<td>1 (2.94)</td>
<td>1 (3.03)</td>
<td>0 (0.00)</td>
</tr>
</tbody>
</table>
4. Discussion

The main finding of this study was the dominancy of genotypes 3a and 1a among groups of patients in different exposure categories. On the other hand, the frequency of genotypes 3a and 1 were 51.1% and 35.6% in over all. The distribution of HCV genotypes in our multiply injected subjects is similar to those that previously reported from Iran[11-14] which shown prominence of genotype 3a and 1a while 1b and 2 are less frequent and other genotypes (4 and 5) are rare in HCV–RNA positive individuals[13,15]. Meanwhile, other studies have reported more frequency of genotype 1a and 3a in thalassemia[16,17]. HCV genotypes distribution in our subjects with different exposure categories is similar to the reported pattern on some HCV high risk population in northern Europe, where genotypes 1, 2 and 3 are more frequent[18,19]. In Iran, variations of HCV infection prevalence have been reported, depending upon the geographical region. In a population–based study from different parts of Iran, Amini et al reported that 1a, 3a, and 1b were the predominant genotypes[11]. On the other hand, since the main subjects of our study population originated from Mazandaran, north of Iran, a prominence 3a genotype which detected in our subjects was in line with other studies shown subtype 3a was more frequent in north of Iran whereas subtype 1a was dominant in south of Iran[13,14,16,20,21]. Distribution of HCV genotypes vary in our neighboring countries. So that genotype 3a was frequently reported form Pakistan[22,23], India[24], and UAE[25] and genotype 1 was more prominent in Turkey[26] and Russia[27]. Furthermore, because of differences in geographical distribution of HCV genotype even in the same country due to distribution of different at risk population, genotyping is an important tool not only for investigating the origin of the HCV outbreak but also for determining the response to interferon therapy, the progression of liver disease, and the outcome of HCV infection. Increasing in prevalence of genotype 3a in our study population have several messages such as HCV genotypes seems to be slightly changed, the potential implications of HCV genotyping in patient selection for appropriate HCV treatment as individuals infected by this genotype have more chance to respond to combination therapy. On the other hand, patients with HCV genotype 3a are more likely to response to routine anti viral therapy while infected patients with genotype 1 may need approximately 2 times longer anti viral treatment. Interestingly, approximately 11.9% of our participants infected with more than one distinct genotype indicated possibility of the new infection. Furthermore, the most frequent mixed genotypes were 1a and 3a. Infection with two or more distinct HCV genotypes was reported in some studies with higher prevalence rates in multiple exposure groups such as hemophiliacs, patients on chronic hemodialysis, and IDAs. Mixed viral infection is of great clinical importance as it may result in more severe disease, failure to antiviral treatment or relapse of infection after the completion of antiviral therapy course[28-30]. Although HCV genotype pattern of our participants are similar to western countries, infection of our subjects with two or more HCV genotypes is an exception. Therefore, it might be due to delay in applying anti–HCV screening of blood products in Iran, which started from 1996[31], in comparison to other countries, multiple exposures to HCV in our high potential risk groups and resultant reinfection of previously HCV–infected patients.

Intravenous drug addicts were more vulnerable to infected to genotype 1 and subtype 1a and 1b. These findings are in contrast to studies in Europe and North America shown a dominant 3a genotype in IDAs[32]. Our result is resemble to studies from Puerto Rico[33] and Brazil[34,35] showed 1a and 1b and the less turn 3a were more frequent in IDAs. Genotypes 1a and 3a are nominated as the IDAs–associated genotypes. It is noteworthy that the most (8.2%) mixed HCV genotypes detected in our participants were 1a and 1b which found in IDAs. Since IDAs become infected with HCV in the early phases of drug addiction and most become exposed repeatedly to the virus through needle–sharing, it is expected that infection with more than one genotype would be common[30,32,36]. It might be a public health threat because of genotype 1b is reportedly associated with a more severe liver disease such as chronic active hepatitis or cirrhosis, and with a poorer response to treatment with interferon than other types of HCV genomes[37]. Since the addiction age is decreasing in Iran and a lot of addicts are IDAs, it might change the subtype pattern of HCV in general population. On the other hand, it has demonstrated that some countries of Europe, genotype 1a and 3a are increasing, while 2a, 2c and 1b are decreasing, especially in young patients[18].

In conclusion, this study is the first report on HCV genotypes among Iranian subjects with different exposure categories resided in Mazandaran, where genotype 3a was found to be the most frequent genotype in thalassemia, hemophilia and HD patients but not in IDAs. Intravenous drug addicts were more vulnerable to infect to subtypes 1a and 1b. Since the addiction age is decreasing in Iran and a lot of addicts are IDAs, it might change the subtype pattern of HCV in general population.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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