Tracing the Spread of Hepatitis C Virus in Turkey: A Phylogenetic Analysis

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Key Words
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Abstract
Background/Aims: Molecular epidemiology of hepatitis C virus (HCV) shows that HCV genotypes are unique with respect to their nucleotide sequence, geographical distribution and clinical relationship.

Methods: In this study we enrolled 67 HCV-infected individuals with various stages of liver disease from four geographical regions of Turkey. A partial NS5B region of the HCV genome was sequenced and subjected to phylogenetic analysis to determine the circulating HCV genotypes and subtypes.

Results: The results showed that HCV genotype 1 (subtype 1b) is the main genetic variant of HCV in Turkey but did not reveal any Turkish indigenous phylogenetic cluster. Phylogenetic analysis showed that Turkish strains have their closest matches from both Asia (Japan) and Europe/USA.

Conclusions: In view of Turkey’s geographic position, HCV-1b transmission from Europe is not exceptional. This study could not establish a clear role of other HCV genotypes prevalent in neighboring Asian countries in Turkey’s HCV transmission, which would need to be confirmed by further regional epidemiological studies.

The Study

Hepatitis C virus (HCV) infection causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma and is responsible for approximately 250,000–350,000 deaths annually worldwide [1]. HCV is a genetically diverged virus and different genotypes of the virus have been associated with distinctive geographical and epidemiological features, as well as with different infection outcomes [2]. The most effective current standard of care in patients with chronic hepatitis C is a combination of pegylated interferon-alfa (PEG-IFN) with ribavirin (RBV). However, in the USA and Europe, only 42–52% of patients with HCV genotype 1 achieve a sustained virological response [3–5] and similar results have been reported in a relatively older Japanese population. Accumulated data has provided strong evidence that approximately 20% of patients with HCV genotype 1, and 4 and 5% of patients with genotype 2 or 3 have a null virological response to PEG-IFN/RBV.

Sequencing of an appropriate region of viral genome, such as the non-structural 5B region (NS5B), core, and E1, is sufficient for discriminating HCV types and subtypes [6]. Phylogenetic analysis of HCV sequences revealed over 70 different subtypes and six large groups of
viral genotypes distributed worldwide [7]. Among those, genotypes 1, 2 and 3 and their subtypes have a global distribution, whereas other genotypes have more local distribution, such as genotype 4 that is found in the Middle East and Africa, genotype 5 in South Africa, and genotype 6 mainly in Asia [7]. Information on different genotypes can have epidemiological value by revealing specific features of local HCV epidemics [8, 9]. However, little is known about regional distribution and genetic features of HCV in Turkey. The aims of this study were to find out the most prevalent HCV genotypes in different regions of Turkey and trace out the HCV transmission patterns.

A total of 67 patients were enrolled in this study. These patients tested positive for anti-HCV and had a serum level of HCV RNA ≥ 50 IU/ml. The data was collected from six medical centers in four different Turkish cities. The patients were classified into three clinical groups: (1) patients with chronic liver disease with persistently elevated serum alanine aminotransferase (ALT) levels defined as chronic hepatitis (CH), (2) patients categorized under the liver cirrhosis (LC) group with clinical evidence of cirrhosis, and (3) patients diagnosed with hepatocellular carcinoma (HCC) on the basis of imaging results as well as on elevated serum fetoprotein (AFP) levels (≥400 ng ml⁻¹). The serological and biochemical tests were done at Ondokuz Mayis University. Samples were screened for HBsAg, anti-HBs, anti-HBc IgG, and anti-HCV by Architect (Abbott Diagnostics, USA). The molecular analysis was performed at the Department of Virology & Liver Unit, Nagoya City University, Graduate School of Medical Sciences, Japan. The study was approved by the Ethics Committee, School of Medicine, Ondokuz Mayis University.

Of the patients analyzed, 12 had diagnosed LC and 12 had diagnosed HCC. Comparative characteristics of these categories are summarized in Table 1. There was also an unequal geographical distribution of the patients in each of the groups with most of the HCC patients enrolled from Ankara (8/12, 66.6%) and most of the LC patients (10/12, 83.3%) enrolled in Gaziantep.

Total RNA was extracted from the serum samples using the SepaGene RV-R Nucleic acid extraction kit (Sanko Junyaku Co., Ltd, Tokyo, Japan) in accordance with the manufacturer’s protocol. Viral RNA were reverse transcribed into complementary DNA using SuperScript II RNase H Reverse Transcriptase (Invitrogen Corp. Carlsbad, Calif., USA) and random hexamer primer (Takara

Table 1. Baseline and clinical features of Turkish patients infected with HCV

<table>
<thead>
<tr>
<th></th>
<th>CH (n = 43)</th>
<th>LC (n = 12)</th>
<th>HCC (n = 12)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male (% age)</td>
<td>16 (37.2)</td>
<td>4 (33.3)</td>
<td>6 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>Age</td>
<td>52±10.5</td>
<td>57.2±11.1</td>
<td>65±7.5</td>
<td>0.0002b, 0.05c</td>
</tr>
<tr>
<td>Total protein, g/dl</td>
<td>7.8±0.6</td>
<td>6.5±0.9</td>
<td>6.9±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>4.1±0.5</td>
<td>3.2±0.6</td>
<td>3.1±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Globulin, g/dl</td>
<td>3.7±0.7</td>
<td>3.3±0.6</td>
<td>3.8±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet count, (×10⁻³), mm³</td>
<td>160.1±103.7</td>
<td>82.1±22.5</td>
<td>128.1±85.8</td>
<td>0.01a</td>
</tr>
<tr>
<td>INR</td>
<td>0.98±0.14</td>
<td>1.56±0.34</td>
<td>1.33±0.24</td>
<td>NS</td>
</tr>
<tr>
<td>ALT, IU/l</td>
<td>87±143</td>
<td>65±57</td>
<td>44±34</td>
<td>NS</td>
</tr>
<tr>
<td>AST, IU/l</td>
<td>74±126</td>
<td>74±40</td>
<td>75±57</td>
<td>NS</td>
</tr>
<tr>
<td>GGT, IU/l</td>
<td>62±71</td>
<td>87±100</td>
<td>91±90</td>
<td>NS</td>
</tr>
<tr>
<td>ALP, IU/l</td>
<td>163±105</td>
<td>112±60</td>
<td>133±82</td>
<td>NS</td>
</tr>
<tr>
<td>T-Bil, mg/dl</td>
<td>0.9±1.5</td>
<td>2.3±1</td>
<td>2.2±1.5</td>
<td>NS</td>
</tr>
<tr>
<td>D-Bil, mg/dl</td>
<td>0.4±1.2</td>
<td>0.9±0.6</td>
<td>1.1±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>HCV RNA (×10⁻⁶), IU/ml</td>
<td>3.4±7.6</td>
<td>1.1±0.1</td>
<td>7.9±10.2</td>
<td>0.01c</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. INR = International normalized ratio; AST = aspartate aminotransferase; GGT = \(\gamma\)-glutamyl transpeptidase; ALP = alkaline phosphatase. a CH vs. LC; b HCC vs. CH; c HCC vs. LC.

Fig. 1. Phylogenetic analysis of HCV isolates from different regions in Turkey. Turkish isolates (in bold letters) were subject to bootstrap resampling with all available sequences in the HCV-NS5B region from the EMBL/DDBJ/GenBank database. The closest neighbors used for the phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBL/GenBank.
Shuzo Co., Ltd, Tokyo, Japan) as described previously [10]. Confirmation of the presence of HCV-RNA in the samples was carried out by amplifying the highly conserved 5′-UTR region and HCV genotypes were determined in non-structural (NS5B) region by direct sequencing [11]. HCV RNA in all HCV RNA-positive samples was quantified by real-time PCR as described previously [12] with slight modifications in an ABI7500 FAST system. The detection limit of the assay was as few as 10 copies/ml.

Amplicons obtained in the NS5B region (nucleotides from 8,278 to 8,618) were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, Calif., USA) in an ABI 3100 DNA automated sequencer. The sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GeneBank. Alignments were performed using CLUSTALW [http://clustalw.ddbj.nig.ac.jp/top-e.html] and neighbor-joining tree was constructed [13]. Statistical differences were evaluated by Fisher’s exact probability test and $\chi^2$ test with Yates’ correction where appropriate, using the STATA software version 8.0 (StataCorp. LP, College Station, Tex., USA). Differences were considered significant for $p$ values <0.05.

Analysis of the HCV genotypes within a defined population is a useful epidemiological tool for the study of HCV infection evolution in different geographical regions and risk groups [8]. Direct sequencing is the most accurate method for HCV genotyping, but again the genotype of the 5′-UTR is less informative, since sequence variation between genotype and/or subtypes is greatest in NS5, less in the envelope and the core, and least in the 5′UTR [14, 15]. In this study, samples were collected from different Turkish cities to find the distribution of HCV genotypes in the partial NS5B region and trace the transmission routes by which HCV made inroads and spread in different geographical localities of the country.

This is the first study analyzing molecular evolutionary characteristics of HCV isolates collected from different regions of Turkey. As shown in figure 1, HCV-1b was found as the main subtype prevalent in Turkey. This is the most widespread variant of HCV, known as the most resistant strain to currently available treatment [16] and associated with HCV-related severe liver sequelae. HCC patients were significantly higher in age compared to the non-HCC, as evidenced in previous studies [17, 18]. Patients with LC had significantly low HCV RNA levels and platelet counts compared to patients with HCC and CH, respectively. However, due to the relatively small size of the cohort, concrete conclusions on clinical outcome may not be assumed. Regardless of the original region within Turkey, no regional bootstrap-significant phylogenetic cluster of HCV-1b was found. Phylogenetic analysis also did not reveal an indigenous phylogenetic cluster for Turkey and the closest matches were from both Asia (Japan) and Europe/USA. This indicates that the HCV spread in this country occurred from different sources possibly via different routes, which is in contrast to the single route exponential transmission described in Egypt or two independent waves as described in Japan [19, 20].

Turkey lies at the crossroads of two continents – Europe and Asia – and due to its geographic position, studying HCV epidemiology has its merits. The transmission of genotype 1b from Europe to Turkey is not exceptional. A fair role of HCV strains of genotypes 3, 4, and 6, predominant in neighboring Asian countries, in Turkey’s transmission was obvious but not observed in this study, probably because of the small cohort of patients studied. Therefore, a comprehensive analysis is required across multiple loci of the HCV genome with a good number of samples taken from different regions of Turkey that may give more insight into the evolution of these viruses in Turkey.

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