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Molecular Characterization of a Full Genome Turkish Hepatitis C Virus 1b Isolate (HCV-TR1): A Predominant Viral Form in Turkey

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Abstract. Based on direct sequencing information from 5′UTR and NS5B regions, we identified subtype 1b as a predominant hepatitis C virus genome in Turkey, which affected more than 91% of 79 patients studied. Next, the full genome sequence of a Turkish 1b isolate was obtained by the cloning of polypeptide-encoding region into 7 overlapping fragments. Turkish 1b isolate, which was named HCV-TR1, comprises 9361 nucleotides, including 306 nucleotides of 5′UTR, a single long open reading frame of 9033 nucleotides, and 22 nucleotides of 3′UTR. When compared to HCV 1b polypeptide sequences available at GenBank, the predicted polypeptide displayed a total of 36 amino acid substitutions, of which 16 was specific for HCV-TR1 isolate. Despite these changes, major structural and functional motifs of HCV proteins were maintained in HCV-TR1. In contrast, HCV-TR1 displayed amino acid substitutions in 6 out of 9 major cytotoxic T-cell epitopes. These data suggest that HCV-TR1 encodes functionally intact viral proteins, but it also encodes altered viral epitopes, which may affect host immune-response.

Key words: hepatitis C virus, subtype 1b, HCV-TR1, Turkey, viral epitope

Introduction

Hepatitis C is a major cause of acute hepatitis and chronic liver disease, including cirrhosis and liver cancer. An estimated 175 millions of persons are chronically infected with HCV worldwide and 3–4 million persons are newly infected each year [1]. The striking genetic heterogeneity of Hepatitis C virus is well recognized. This genetic diversity includes six major genotypes, with numerous subtypes (over 80) and minor variants called 'quasispecies' [2]. The geographical distribution of different genotypes and subtypes differs greatly from one region to the other. The reasons for this differential distribution are not well known, but the profile of geographical distribution could reflect the different modes of viral transmission as well as the host immune-response variations. For example, HCV 1a subtype, which is seen frequently in North America, could have been transmitted to other regions of the world, especially to Europe, by contaminated blood-derived products [3]. In contrast, subtype 1b appears to be dominant in Japan and Southern Europe. This particular subtype has been related to more severe liver disease, resistance to interferon treatment and increased risk for hepatocellular carcinoma [4,5].

Turkey with a population of over 65 million people is located between southern Europe and the Middle Eastern countries, which differ from each other by the distribution of major genotypes. In Europe, HCV 1

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is the major genotype and there is a south-north gradient for 1a and 1b subtypes, the prevalence of subtype 1b being increasingly higher in southern Europe [4–7]. The data on HCV subtypes in the Middle Eastern countries is limited. In Egypt, Hepatitis C is an endemic disease that is associated with genotype 4, almost exclusively [8]. Similarly, genotype 4 is also predominant in the Gaza region, but not in Israel where subtype 1b is predominant [9]. The distribution of HCV genotypes in liver disease patients living in Syria, Iraq and Iran is unknown. In one study performed in hemodialysis patients from Syria, genotypes 4a, 1b and 1a were identified in respectively 30%, 27% and 19% of patients, and 30% of sequences were unmatched [10]. On the other hand, in countries located on the northern frontiers of Turkey, subtype 1b appears to be the dominant form [11–13].

The HCV genotype distribution of patients living in Turkey is not well known. To our knowledge, there are only two published reports concerning Turkish patients, which indicated a high frequency (75–87%) of subtype 1b [14,15]. The regional distribution of HCV genotypes in Turkey has not been reported yet. In this study, we report our observations on HCV subtypes in southern Turkey. This region shares its frontiers with Middle Eastern countries displaying heterogenous distribution of subtypes 1a, 1b and 4a of HCV, as stated earlier [8–10,16]. Here, we report that the subtype 1b is the predominant genotype in Hepatitis C patients living in southern Turkey. This confirms that Turkish patients are infected mostly with this particular subtype of HCV. In contrast, subtype 1a was rare, others including subtype 4 being exceptional. Based on this information, we obtained sequence data for the 5′UTR and entire polyprotein coding regions of a Turkish 1b isolate, termed HCV-TR1, following cloning of viral genome into 7 overlapping fragments. We present here the main features of the predicted viral polyprotein sequence of TR1, in comparison with that of other HCV 1b isolates from different geographical regions in the world.

Material and Methods

Patients

A total of 79 HCV-positive patients from the Gastroenterology Department of Çukurova University in Turkey were investigated for HCV genotyping. The great majority of these patients lived in South Anatolia and Southeast Anatolia regions at the time of diagnosis. All patients were positive for anti-HCV antibodies, which were determined using the second-generation ELISA test. Among this group of patients, a serum sample from a 59-year old woman suffering from chronic Hepatitis C, which was collected prior to any treatment for her disease, was used for HCV cloning studies.

Viral RNA Extraction and cDNA Synthesis

We used a modified and optimized RNA extraction protocol derived from a previously published procedure [17]. RNA was extracted from 300 µl of serum with the freshly made lysis buffer containing guanidine-HSCN and mercaptoethanol in the presence of an RNA carrier. The RNA was then recovered by isopropanol precipitation and resuspended in 10 µl DEPC–ddH2O. First strand cDNA synthesis was performed using a commercial kit (MBI). Briefly, 10 µl resuspended RNA was treated in a 20 µl reaction volume with 0.2 µg of random primers, 40 U of M-MuLV Reverse Transcriptase, 20 U of Ribonuclease inhibitor, and 1 mM (each) deoxyribonucleotides at 37°C for 1 h after brief denaturing at 90°C.

PCR Amplification of 5′UTR and NS5B Regions for Genotyping Studies

Initially we used sequence information at 5′UTR for genotyping studies. Sequence information derived from NS5B region was used for confirmation studies. A 285 bp fragment from the 5′UTR of the HCV genome was generated with PCR amplification of one-fourth of cDNA. The first round of 'nested' PCR was performed using outer primers F1 (5′-ATCACTCCCCTGTGAGGAAC-3′) and R1 (5′-TGCTCATGGTGCACGGTCTAC-3′), after denaturation at 94°C for 5 min, with 25 cycles, each containing 45 s at 94°C, 45 s at 55°C and 45 s at 72°C, followed by a final extension for 10 min at 72°C. For the second amplification, 8 µl of the first reaction mixture was further amplified with inner primers F2 (5′-GAGGAACTACTGTCTTCACGC-3′) and R2 (5′-TCTACGAGACCTCCCGGGGCA-3′) under the previous conditions, except the annealing temperature which became 60°C to generate a fragment of 285 bp. For PCR amplification of NS5B region, two-step PCR

with the same primer set was established to generate a 400 bp DNA fragment covering region 7904–8304 (Position of 5′ base relative to HCV genomic sequence in Choo et al.; 1991 [33]) by using NS5B Forward (5′-TGGG-GATCCCGTATGATACCCGCTGCTT-TGA-3′) and NS5B Reverse (5′-GGCGGAA-TACCTGGTCATA-GCCTCCGTGAA-3′) primers. PCR amplification was done after denaturation at 94°C for 4 min, with 30 cycles, each containing 40 s at 94°C, 40 s at 58°C and 40 s at 72°C, followed by a final extension for 10 min at 72°C. For the second amplification, 4 µl of the first reaction mixture was further amplified with the same set of primers in a total volume of 50 µl, using Taq *Polymerase* (MBI). Specific PCR amplification of correct-sized DNA fragments was confirmed by agarose gel electrophoresis and the appropriate amount of PCR products (usually 100 ng) were subjected to automated nucleic acid sequencing using the same sets of primers and cycle sequencing kits from Perkin Elmer and Amersham following the manufacturer's instructions. Sequencing reaction products were analyzed on ABI-377 DNA sequencer (Perkin Elmer).

HCV Genotype Identification and Phylogenetic Sequence Analysis

The 5′UTR and NS5B regions of all available genotypes at NCBI Taxonomy Homepage were aligned using MULTIALIN multiple alignment program to reveal the subtype-specific consensus sequences (http: //npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?-page=/NPSA/npsa_multalinan.html). The same groups of sequences were also used to construct the phylogenetic tree by using the PHYLIP program to determine the genotypic distribution of all samples. In order to compute the distance matrix, we used DNADIST module from PHYLIP software package with Kimura 2-parameter model (http://sdmc.krdl.org.sg:8080/~lxzhang/phylip).

Molecular Cloning and Characterization of a Turkish HCV 1b Isolate (HCV-TR1)

A nearly full length HCV isolate from a 59-year old female Turkish patient was amplified in 7 overlapping PCR fragments. Primer sets for each fragment, which were designed from the most conserved regions in the desired area, are given in Table 1. Fragments smaller than 1 kb were amplified with *Pfu* DNA *Polymerase* in the first round in order to decrease the PCR-mediated mutation risk and in the second round *Expand High fidelity PCR System* (Boehringer Mannheim) was used to obtain PCR products with adenine overhangs. Fragments larger than 1 kb were amplified with *Expand High Fidelity System*. PCR products were cloned into pGEM®-T Easy vector (Promega), which is a linearized vector with T overhangs in the multiple cloning site. Selected plasmids containing desired inserts were purified by using QIAfilter plasmid midi kit (Qiagen), and used for automated DNA sequencing as described. For sequencing,

Table 1. Sequences of primers used for PCR amplification of overlapping cDNA regions of the genome of HCV isolate HCV-TR1

Primer	Sequence (5′ → 3′)	Position*	Amplified Fragment
F1	ATCACTCCCCTGTGAGGAAC	−306	5′UTR and Core
CoreR	(G/A)GAGCA(G/A)TCGTTCGTGACAT	964	
E1F	CCCGGTTGCTCTTTCTCTATC	850	E1, E2, and p7
E2R	ATGC(A/G)GCCATCTCCCGGTC	2791	
NS2F	T(C/T)CT(G/A)(C/T)TG(G/T)C(G/A)TTACCACC	2738	NS2
NS2R	GT(C/T)TG(C/T)TG(G/A/T)G(A/C)GTAGGCCGT	3449	
NS3F	CCGAAGGGGGA(A/G)GGAGAT	3354	NS3
NS3R	GCACCCA(G/A)GTGCT(A/C/G)GT(G/A)ACGAC	5326	
NS4F	ATGCATGTCGGC(C/T)GACCT	5283	NS4A and NS4B
NS4R	TG(G/A)AGCCA(G/A)GTCTTGAAGTC	6329	
NS5AF	TATGTGCCTGAGAGCGACG	6142	NS5A
NS5AR	(A/G)CG(C/T)AGCAAAGAGTTGCTCA	7695	
NS5BF	AGCGACGGGTC(C/T)TGGTCTAC	7543	NS5B and 3′ UTR
NS5BR	CCTGGAGTG(G/T)TT(A/G)GCTCCC	9397	

*Nucleotide numbers according to Ref. 33.

plasmid-derived primers were used initially, followed by HCV sequence-derived sense and anti-sense primers. Sequence data obtained from overlapping fragments was assembled manually to construct a full length HCV genome sequence.

Results

Hepatitis C Virus Genotyping in Turkish Patients

A total of 79 HCV RNA-positive sera from patients living in southern Turkey were used for genotyping. The amplified 5′UTR region was analyzed by automated DNA sequencing. The sequence data was compared with HCV subtype-specific consensus sequence data as described in the section 'Materials and Methods'. A phylogenetic tree for HCV subtypes in our sample population was constructed in comparison with consensus HCV genotypes. For the analysis, a 100-base region located between nucleotides −172 and −72 of HCV was selected, since this region was variable enough between HCV subtypes. Out of 79, 70 samples displayed unambiguous nucleotide sequence at this region. The sequence similarities between our sample population ranged from 84 and 100%. In order to build the phylogenetic tree, we included 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b and 4c subtype consensus sequences into our population data, and we examined the distances between sequence clusters. Prior to this analysis, we calculated sequence similarities between subtype consensus sequences, which ranged from 75 (between 2a and 3b) to 99% (between 1a and 1b). The phylogenetic tree indicated that out of 70 nucleic acid sequences, 3 were clustered with 1a subtype and displayed 0–0.0102 evolutionary distances. Sixty-five sequences were grouped together with 1b subtype showing a distance rate of 0–0.0219. One sequence was grouped with 2a subtype with a zero evolutionary distance, while another sequence grouped with subtype 4c consensus with zero evolutionary distance. There was no sequence clustered with 2b, 3a, 3b and 4a subtypes (Fig. 1). This phylogenetic tree analysis showed that 5′UTR data can be used for subtype identification. Accordingly, the remaining 9 samples were genotyped by manual alignment with subtype-specific consensus sequences. To confirm HCV genotyping results obtained by 5′UTR sequence data, 19 randomly selected samples were genotyped using NS5B region sequence data and all samples displayed the expected genotype (data not shown). When combined, these analyses indicated that, 72 of 79 patients (91%) displayed 1b genotype of HCV, and 5/79 (6%) had genotype 1a. Thus, all but 2 of 79 HCV genotypes analyzed had genotype 1. The other two samples displayed subtypes 2a and 4c, respectively.

Molecular Characteristics of Turkish Isolate of HCV 1b (HCV-TR1)

Following our identification of subtype 1b as the main HCV genotype affecting Turkish patients, we decided to obtain full genome sequence information from a Turkish HCV 1b isolate. Using a single serum sample obtained from a patient prior to any treatment, we cloned the major portion of HCV genome into 7 overlapping fragments. When combined together, these clones covered the entire sequence for HCV polyprotein, as well as most of the nucleotides of the 5′UTR and 3′UTR regions. The overlapping regions all contained the identical sequences. The Turkish 1b isolate, which we named HCV-TR1, comprises 9361 nucleotides, including 306 nucleotides of 5′UTR, a single long open reading frame of 9033 nucleotides, and 22 nucleotides of 3′UTR (data submitted to GenBank nucleotide sequence database and assigned the accession number AF483269). This genomic sequence showed highest homology (91% identity) to a reported HCV 1b isotype (strain HCV-1b, clone HCV-K1-R2) from Japan [18], when tested by BLAST using GenBank database. The HCV-TR1 displayed a single open reading frame encoding a 3010-amino acid polyprotein that showed 93% identity to clone HCV-K1-R2.

Proteolytic processing of HCV polypeptide between C-E1, E1-E2, E2-p7 and p7-NS2 is performed by host cell proteases, whereas viral proteases cleave between the non-structural proteins NS2-NS3, NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B [19]. HCV-TR1 polypeptide displayed no major amino acid change in boundary regions at proteolytic cleavage sites (data on cleavage sites of non-structural proteins are shown in Table 2), suggesting that it can be correctly processed into HCV proteins, including core (1–191; 191 aa), E1 (192–383; 192 aa), E2 (384–746; 363 aa), p7 (747–809; 63 aa), NS2 (810–1026; 217 aa), NS3 (1027–1657; 631 aa), NS4A (1658–1711; 54 aa), NS4B (1712–1972; 261 aa), NS5A (1973–2419; 447 aa), NS5B (2420–3010; 591 aa) proteins. Because of nucleic acid

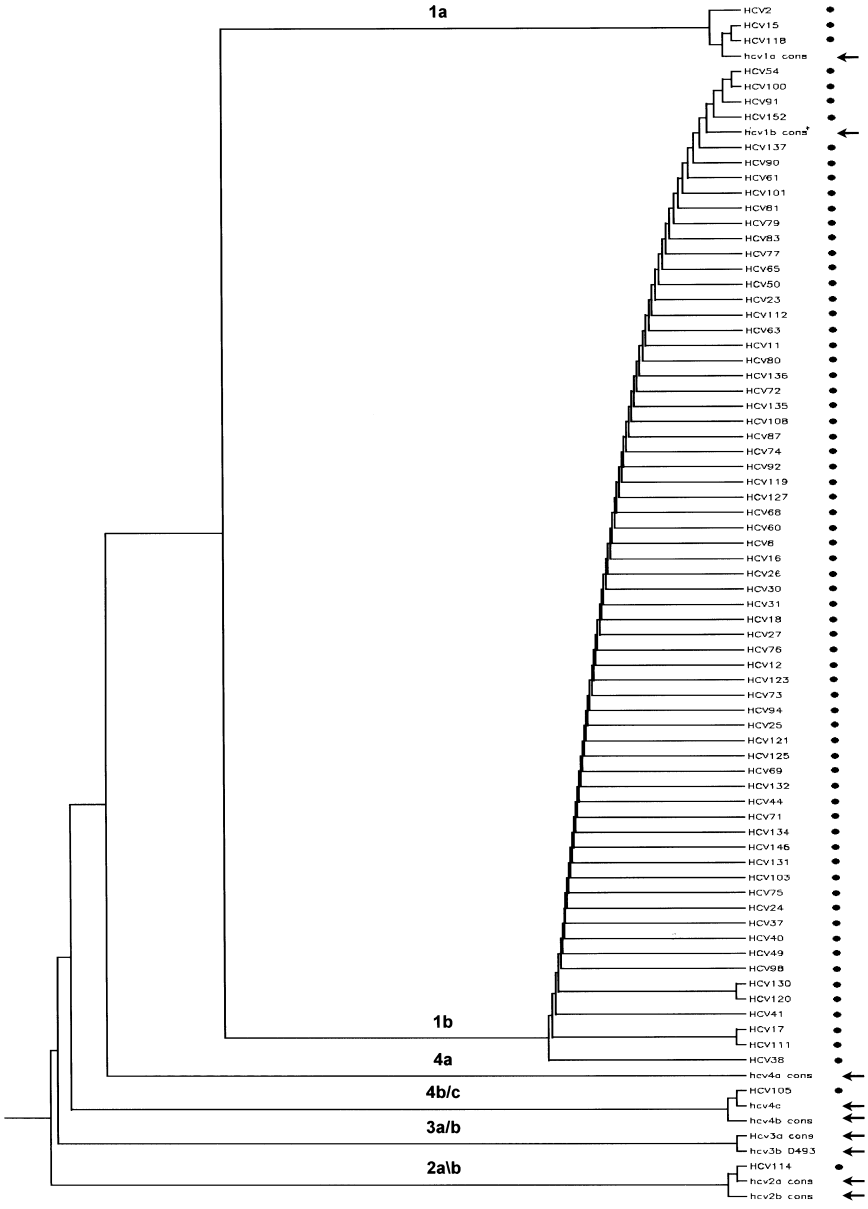

Fig. 1. Phylogenetic tree of the 5′UTR sequences from 70 isolates (filled circles) of HCV from Turkey. The consensus sequence data for subtypes 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b and 4c (arrows) were included as reference sequences. The genetic distances were calculated with the DNADIST module from PHYLIP software package and were based on a Kimura 2-parameter matrix with a transition to transversion ratio of 2 (see text).

sequence ambiguities, we were not able to determine the amino acid residue in 3 positions. The alignment of this polyprotein sequence with that of other 48 HCV-1b isolates available at the GenBank database revealed that there was overall 36 amino acid substitutions in the Turkish isolate. Twenty of these substitutions occurred at amino acid residues that showed variations among different 1b isolates. In contrast, the remaining 16 amino acid changes of HCV-TR1 occurred at conserved amino acid residues.

Table 2. Comparison of inferred amino acids at proteolytic cleavage sites between HCV-TR1, HCV-J and HCV-BK

Genotype	NS2/NS3
HCV-TR1	ADSFKGQGWRLL ↓ APITAY
HCV-J	ADSFGEQGWRLL ↓ APITAY
HCV-BK	ADSLEGRGLRLL ↓ APITAY
	NS4A/NS4B
HCV-TR1	ALYQAFDEMEEC ↓ ASHLPY
HCV-J	VLYQEFDEMEEC ↓ ASHPLY
HCV-BK	LLYQEFDEMEEC ↓ ASHPLY
	NS5A/NS5B
HCV-TRI	SEEASEDVCCC ↓ SMSYTW
HCV-J	SGEAGEDVVCC ↓ SMSYTW
HCV-BK	SEEASEDVVCC ↓ SMSYTW
	NS3/NS4A
HCV-TRI	MACMSADLEVVT ↓ STWVLV
HCV-J	MACMSADLEVVT ↓ STMVLV
HCV-BK	MACMSADLEVVT ↓ STMVLV
	N4SB/NS5A
HCV-TRI	HQWINEDCSPTC ↓ SGSWLR
HCV-J	HQWINEDCSTPC ↓ SGWLK
HCV-BK	HQWINEDCSPTC ↓ SGSWLR

↓, denotes cleavage sites. Amino acid residues of HCV-TR1 that are different from that of two other HCV-1b isolates [34,35] are underlined.

Out of 16 unique amino acid substitutions, half were conserved, the other half not being conserved (Table 3).

Main Features of HCV-TR1 Polyprotein Primary Structure

When compared to consensus HCV 1b polypeptide sequence, ten conserved cystein residues (14, 89, 170, 223, 243, 274, 279, 295, 366, 521) of HCV [20], five N-glycosylation motifs (NXS) of E1 protein [21], the catalytic triad (His_{57}, Asp_{81}, Ser_{139}), AX_4GKS motif involved in ATP binding, as well as Cys_{97}-Cys_{99}-Cys_{145}-His_{149} metal binding site of NS3/NS4A heterodimeric serine protease [22–24] were conserved. In addition, motifs I (207-GXGKS/T-211), II (290-DECH-293), III (322-TATPP-326), V (410-ATDALMTGFTGDFD-423), VI (460-QRXGRXGR-467) and putative Motif IV (370-SK-371) of NS3 helicase were maintained in HCV-TR1 [23–25]. The ISDR (Interferon Sensitivity Determining Region) of NS5A (2209-PSLKATCTTHHDSPDADLIEANLL-WRQEMGGNITRVESEN-2248) was also conserved [26]. The HVRI region, which is the most prone to mutation in HCV, was also maintained for 25 of 27 residues in HCV-TR1 [27]. These comparative data indicates that viral proteins encoded by HCV-TR1 share the main structural and functional features with other HCV isolates.

Table 3. Summary of amino acid differences between the HCV-TR1 Turkish isolate and other characterized HCV-1b genomes

Region	Amino Acid Position	Other HCV 1b isolates[a]	HCV-TR1	Type of Substitution[b]
E2	591	E	D	C
E2	728	D	Y	NC
E2	750	N	I	NC
NS3	1075	N	S	C
NS3	1539	L	V	C
NS3	1628	L	M	C
NS4A	1704	E	A	NC
NS5A	2170	V	T	NC
NS5A	2176	T	I	NC
NS5B	2493	K	E	NC
NS5B	2556	T	V	NC
NS5B	2567	Q	K	C
NS5B	2570	K	M	NC
NS5B	2838	L	I	C
NS5B	2897	S	P	C
NS5B	2996	L	F	C
Core	187	I, T, V	M	
E2	397	F, G, H, I, L, M, Q, R, S, Y	A	
E2	478	D, G, H, N, Q, R, S, V	E	
E2	493	P, Q, R	K	
E2	580	I, T	L	
E2	626	I, L	V	
NS2	828	F, L	I	
NS2	857	L, M	V	
NS2	861	I, V	T	
NS2	949	I, V	L	
NS3	1290	P, S	G	
NS3	1382	I, L	V	
NS3	1636	I, T, V	N	
NS5A	2030	P, Q	S	
NS5A	2079	S, F	T	
NS5A	2302	K, R	E	
NS5B	2554	D, N, Q	E	
NS5B	2617	G, K	A	
NS5B	2665	A, S, V	T	
NS5B	2736	D, N	S	

[a]Data obtained from 48 HCV 1b isolate amino acid sequences available at the GenBank database.

[b]Conserved (C) and non-conserved (NC) amino acid substitutions at residues conserved in all published sequences were based on PAM250 amino acid scoring matrix [36].

Table 4. Comparisons of immunodominant Cytotoxic T cell epitopes of HCV with corresponding amino acid residues in Turkish isolate HCV-TR1

Viral Protein	Amino Acid No.	Epitope[a]	HCV-TR1[*]
Core	178–187	LLALLSCLTV	LLALLSCLT**M**
E2	402–412	SLLAPGAKQNV	SL**F**A**S**G**PT**Q**RI**
NS3	1073–1081	CINGVCWTV	C**VS**G**A**CWTV
	1406–1415	KLVALGINAV	KL*S*G*L*G*L*NAV
NS4B	1671–1680	VLAALAAYCL	VLAALAAYCL
NS5A	1992–2000	VLSDFKTWL	VLSDFKTWL
	2145–2154	LLREEVSFRV	LLREEVSFRV
	2221–2231	SPDAELIEANL	SPDA**D**LIEANL
NS5B	2594–2602	ALYDVVTKL	ALYDVV**S**TL

[a]From Refs 28 and 29.
[*]Amino acid residues of HCV-TR1 that are different from the known epitope are underlined.

The HCV virus was shown to harbor several Cyotoxic T-Cell (CTL) and T-Helper Cell-specific dominant epitopes that may play a major role in host immunity toward viral infection [28,29]. Table 4 shows immunodominant CTL epitopes of HCV, in comparison with corresponding amino acid residues of HCV-TR1. There are 8 known major CTL epitopes of HCV. Among these, one epitope located on NS4B protein (aa 1671–1680), and 2 epitopes located on NS5A protein (aa 1992–2000 and 2145–2154) were fully conserved in HCV-TR1. In contrast, HCV-TR1 displayed amino acid substitutions in the five remaining epitopes located on core, E2, NS3, NS5A and NS5B proteins, respectively. Of particular interest, 6 out of 11 (55%) amino acid residues of an E2 epitope (aa 402–412) were different in HCV-TR1. Similarly, two NS3 epitopes (aa 1073–1081 and 1406–1415, respectively) displayed amino acid changes in three positions (30–33% difference). In contrast to high rate of mutations in CTL epitopes, the dominant T-Helper Cell-specific epitope located on NS3 protein (aa 1251–1259; VLVLNPSVA) was conserved in HCV-TR1 [28].

Discussion

The first observation of this study was that a 100 bp sequence region (−172 to −72) of HCV 5′UTR carries enough sequence variations for differential analysis of 9 subtypes of genotypes 1 through 4, as shown in Fig. 1. The phylogenetic analysis also showed that the HCV isolates identified in Turkish patients did not diverge from other known and commonly found HCV isolates. Indeed, the great majority of these isolates (91%) were identified as subtype 1b with a maximum evolutionary distance of 0.0219 within this group. The subtype 1a was rare (6%), while genotypes 2 and 4 were exceptional (1/79 for each case). Thus, more than 90% of HCV infections in Turkish patients living in the southern region are caused by a single subtype, namely 1b. Our results confirm earlier reports for Turkish patients that showed 1b as a predominant subtype in other regions of Turkey [14,15]. Thus, it appears that HCV infections in Turkey are due almost exclusively to a single subtype, namely subtype 1b. The predominance of 1b subtype in Turkey correlates with the north-south gradient of increased 1b subtype occurrence in Europe. For example, a similarly high frequency of 1b subtype (91%) was reported for Sicilian patients, while only 8% of HCV infections in Finland are due to the same subtype [30]. It was hypothesized that HCV infections with 1a subtype in Europe are due to the use of blood products originating from the USA. The low prevalence of 1a subtype in Turkey may support this hypothesis and indicates that HCV infections in Turkey are due to the local propagation of a 1b subtype. The exceptional occurrence of genotype 4 in southern Turkey is in favor of such a hypothesis. This particular genotype is endemic in Egypt and highly prevalent in the other Middle East countries with the exception of Israel [8,9]. It appears that the genotype 4 did not propagate from these countries towards Turkey.

After identification of subtype 1b as the predominant genotype, whole genome sequencing of a Turkish HCV-1b isolate from a single human carrier was performed. When compared to other variants of 1b subtype, the Turkish HCV-1b isolate displayed highest homology to a Japanese 1b strain. The reasons for this close relationship between Turkish and Japanese HCV isolates are presently unknown. As reported by Smith et al. [31], the average time of divergence of variants of subtype 1b was about 70–80 years ago. In addition, the absence of country-specific groupings by phylogenetic analysis of subtype 1b sequences suggested that the spread of this genotype occurred on a worldwide basis at a similar time [32]. Thus, the high homology of a Turkish isolate (HCV-TR1) with a Japanese isolate provides further evidence for this prediction. The HCV-TR1 polyprotein displayed amino acid substitutions at 36 positions when compared to other 1b variants. More than 50% of these substitutions occurred at residues that were hetero-

genous among different isolates. However, HCV-TR1 displayed specific changes in 16 positions and 8 of them were non-conserved amino acid substitutions (Table 3). Presently, it is unknown whether such changes affect functions of concerned viral proteins, however none of them appear to affect previously known functional motifs of structural and non-structural HCV proteins. Therefore, it appears that the Turkish HCV-TR1 share similar features with other HCV isolates in terms of amino acid residues directly involved in protein function.

In contrast to the conservation of functional characteristics of viral proteins, a high number of immunodominant epitopes of HCV-TR1 displayed structural changes. Of particular interest, three CTL epitopes, one located on E2 and two on NS3 viral proteins displayed a high rate of amino acid substitutions (30–55%). Based on the fact that major functional features of HCV proteins are conserved, but many CTL-epitopes displayed substitutions at several amino acid residues, we believe that mutations affecting immunodominant viral epitopes in HCV-TR1 are not due to experimental errors and represent true changes in the immunogenicity of this strain.

HCV-specific CD8 + cytotoxic T lymphocytes are believed to play an important role in the pathogenesis of liver cell injury and viral clearance in HCV infection [28]. The efficacy of anti-viral cytotoxic immune response relies on the availability of viral epitopes to be recognized by specific CTLs. The fact that 6 out of 9 major CTL epitopes of HCV-TR1 are different from the consensus epitope sequence (Table 4), strongly suggests that the host immune response to this viral strain is defective or deficient. Further studies are needed to know whether mutations affecting immunodominant CTL epitopes may serve as a basis for unusually high frequency of HCV infections with 1b subtype in Turkish patients. More importantly, immunodominant CTL epitopes are considered as candidates for design of therapeutic vaccines for HCV [29]. Such vaccines may not be efficient against strains such as HCV-TR1 since they display major amino acid changes at candidate vaccine epitopes.

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