Prevalence and Genotypes of Hepatitis G Virus among Hemodialysis Patients in Eastern Anatolia, Turkey

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Key Words

Hepatitis G virus · Hemodialysis patients · Genotypes · East Anatolia, Turkey

Abstract

Objectives: To study the prevalence and genotype distribution of hepatitis G virus (HGV) in hemodialysis patients in East Anatolia, Turkey. **Subjects and Materials:** Eighty-nine hemodialysis patients and 30 healthy individuals were analyzed by using reverse-transcriptase nested polymerase chain reaction with primers specific for 5' untranslated region. HGV genotyping was performed by PCR and three randomly selected HGV-positive samples were sequenced. **Results:** Of the 89 hemodialysis patients, HGV RNA was detected in 9 (10.2%). All of our isolates were assigned to genotype 2. **Conclusion:** Our results showed that hemodialysis patients carry the risk for HGV infection in East Anatolia, Turkey.

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Introduction

Hepatitis G virus (HGV) is a recently described member of flaviviruses. The genome of the virus consists of single-stranded RNA and has a positive polarity. Similar

to other members of the flaviviridae, it contains a single open reading frame that encodes the viral polyprotein [1, 2]. The natural history of HGV infection is at present not fully understood. The potential of HGV to cause hepatitis in humans is questionable. Controversial data exist concerning whether or not HGV replicates in the liver [3–6].

HGV is highly prevalent among population groups at risk of parenterally transmitted viral agents, but it has also a worldwide distribution in other non-risk population groups. Hemodialysis (HD) patients have a high risk of blood-borne viral infections [7]. Some of the most common viral infections are caused by hepatotropic or other hepatitis-associated viruses such as hepatitis B virus (HBV) [8], hepatitis C virus (HCV) [9], HGV [10] and transfusion-transmitted virus [11]. In recent years, a high prevalence of HGV infection among end-stage renal disease and chronic HD patients has been well documented, varying between 6 and 44%, in different countries [12–15]. Analysis of the 5' untranslated region (UTR) has led to the suggestion that variants of HGV can be divided into five genotypes [16]. Genotype 1 is prevalent in West and Central Africa; genotype 2 in Europe, North America and East Africa, Pakistan and Japan; genotype 3 in parts of Asia; genotype 4 in South-East Asia, and genotype 5 in South Africa [16, 17]. Investigation of genetic diversity among sequences of different isolates of a virus

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1011–7571/05/0142–0102\$22.00/0

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Table 1. Oligonucleotides used for amplification and genotyping of *HGV* in this study

Primer	Sequence specificity ¹	Sequence polarity
HG1	5'-GGTCGTAATCCCGGTCACC-3'	universal/sense
HG1R	5'-CCCACTGGTCCTTGTCAACT-3'	universal/antisense
HG2R	5'-ATTGAAGGGCGACGTGGACC-3'	universal/antisense
Mix A		
G38	5'-TGTAATAAGGACCCGGCGMT-3'	type 1-specific/sense
G41	5'-TGGTCAAGGTCCTCTG-3'	type 3-specific/sense
Mix B		
G35	5'-GGGTCTTAAGAGAAGGTTAAGA-3'	type 2-specific/sense
G40	5'-GGGTYAAGGCACCTCTTA-3'	type 4-specific/sense

¹M = A or C; Y = C or T.

has become important because variants show different patterns of virulence, reactivity, and response to antiviral therapy [10, 17]. The present study was undertaken to determine the prevalence and genotype distribution of HGV in HD patients in Elazig, East Anatolia, Turkey.

Subjects and Methods

Subjects

We studied 89 patients (46 men and 43 women; mean age 49.4 ± 17.6 years) with renal failure who were undergoing HD at the government hospital hemodialysis units. The patients had been on maintenance HD for a mean period of 89.7 ± 15.3 weeks (range, 2–728 weeks). Thirty healthy individuals (18 men and 12 women; 42.4 ± 19.6 years) who did not belong to any risk group for viral hepatitis served as negative controls. Sera from the HD patients and the control group were tested for markers of HBV and HCV infections, and serum levels of alanine aminotransferase (ALT) were measured. Blood samples were collected and centrifuged immediately and stored at -80°C until used. The study was approved by the Ethics Committee of the institution, and all patients gave informed consent.

Serological Markers of Hepatitis B and C Infections, and Serum ALT Values

HBV surface antigen (HBsAg) was detected with commercially available enzyme-linked immunosorbent assays (ELISA, Abbott Diagnostics, Dartford, UK). Detection of anti-HCV antibody was measured using commercially available second-generation ELISA (Abbott Diagnostics). The serum ALT values were measured by using Olympus AU 600 autoanalyzer (Olympus Corp., Japan). The level of ALT has an upper reference range of 40 U/l.

Determination of HGV by Reverse Transcription Nested Polymerase Chain Reaction (RT-n-PCR)

RNA was obtained from serum by EZ-RNA Total RNA Isolation Kit (Biological Industries Co. Beit Haemek). RT was performed by incubating the template (approximately 100 μl of serum) in the presence of 50 pM outer antisense primer (HG1R; nt 381 to

400) and 100 U of Moloney murine leukemia reverse transcriptase (Promega, Madison, Wisc., USA) for 1 h at 37°C . PCR amplification of cDNA was carried out in a 50- μl volume by using HG1R and outer sense primer (HG1; nt 139 to 158). After a denaturation step (5 min at 94°C), 33 cycles of PCR followed with one cycle consisting of denaturation (30 s 94°C), annealing (1 min 55°C) and primer extension (1 min 72°C). Nested PCR was performed in a 50- μl volume after transfer of 2 μl from the first round of PCR to a mix containing 50 pM inner sense primer (HG2; nt 163 to 182) and an inner antisense primer (HG2R; nt 331 to 350). The nested PCR was performed after a denaturation step as given above except that 40 cycles instead of 33 cycles were run. Amplicons of 188 bp were expected. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide and visualized under UV light.

HGV Genotyping by PCR

HGV genotyping by PCR was performed as described previously [18]. Briefly, the RNA was extracted from 100- μl serum samples. The 5' UTR region of the HGV genome was amplified with universal primers (HG1 and HG1R). Then, two second-round PCRs were carried out for each sample, with the common universal antisense primer (HG2R) and two different mixtures (mix A and B) including type-specific sense primer pairs shown in table 1. A 2- μl aliquot of the first PCR product was subjected to two tubes containing the second sets of each of the inner primer pairs, deoxynucleotides, Taq DNA polymerase and PCR buffer, and the same PCR parameters as in the first reaction. To distinguish genotype 2 from genotype 4 by agarose gel electrophoresis, we amplified an amplicon in 161 bp length, which is the same size as genotype 4, as a marker, using pSV2-Cat cloning vector (accession number M777788) as a template with primer 1 5'-AATGTTGAGAGT-CAGCAGTAGCC-3' and primer 2 5'-GAATCAGTA GTTTAA-CACAT-3' that bind to pSV2-Cat cloning vector at positions 4450 and 4611, respectively. Amplified products were visualized after electrophoresis through a 3% agarose gel stained with ethidium bromide.

Nucleotide Sequence of HGV Isolates

To confirm the validity of the HGV genotyping system by PCR, three amplified DNA fragments were subcloned into pGEM-T vector (Promega, Madison, Wisc., USA) before being subjected to se-

Fig. 1. PCR-based genotyping of HGV of the isolates. Lanes 1, 2 = RT-n-PCR-positive samples (188 bp); lanes 3, 4 = two isolates of genotype 2 (183 bp); lanes 5, 6 = 161-bp amplicon amplified with P1 and P2 primer; M = 50-bp DNA ladder (Promega).

quence analysis. Cycle sequencing was done by the chain termination method using fluorescent dye termination (ABI PRISM™, Foster City, Calif., USA). Products were separated in an automated DNA genetic analyzer (ABI 310 Prism Perkin-Elmer Corp., Foster City, Calif., USA). The nucleotide sequences were deposited in the EMBL/GenBank databases under the accession numbers AY324428, AY324429 and AY324430. Sequences were compiled and aligned using Clustal W (v1.8). The phylogenetic tree was generated comparing the study sequences with earlier reported sequences using the neighbor-joining method in the Treecon program.

Statistical Analysis

The Fisher's exact and Mann-Whitney U tests were used for statistical evaluation. The values of $p < 0.05$ were accepted as statistically significant.

Results

HGV RNA was detected in 9 (10.2%) of the 89 HD patients by RT-n-PCR (fig. 1). Table 2 shows the characteristics of the 89 HD patients, according to their HGV RNA status. Anti-HCV (in 6 patients, 6.7%) and HBsAg (in 1 patient, 1.1%) were also detected. Of the 9 patients with HGV RNA, 2 were also seropositive for anti-HCV and but not for HBsAg. Thus, 22.2% of the patients with HGV infection were also seropositive for anti-HCV while the 30 healthy individuals were negative for HGV RNA, anti-HCV and HBsAg. The mean duration of HD therapy in the HGV RNA-positive patients (168.2 ± 50.1 weeks) was significantly longer than in the HGV RNA-negative patients (79.9 ± 15.9 weeks, $p < 0.05$). The

Table 2. Characteristics of 89 HD patients, according to their HGV RNA status

Patient characteristics	Findings in patients with HGV RNA		p value
	positive	negative	
Patients	9 (10.2%)	80 (89.8%)	<0.05
Age, years (mean $\pm$ SD)	49 ± 21.08	49.4 ± 17.3	NS
Male patients	3 (33.3%)	43 (53.7%)	NS
Female patients	6 (66.7%)	37 (46.3%)	NS
Weeks on HD (mean $\pm$ SD)	168.2 ± 50.1	79.9 ± 15.9	<0.05
Blood transfusions	9 (100%)	80 (100%)	
Renal transplantations ¹	–	–	
Patients with elevated ALT	3 (33.3%)	2 (2.5%)	
ALT levels, U/l	54 ± 21.6	15.2 ± 1.2	<0.05
Anti-HCV			
Positive patients	2 (22.2%)	4 (5%)	NS
Negative patients	7 (77.8%)	76 (95%)	
Serum HBsAg ²			
Positive patients	–	1 (1.2%)	NS
Negative patients	9 (100%)	79 (98.8%)	

NS = Nonsignificant.

¹A minus sign denotes patients who did not receive renal transplantation.

²A minus sign denotes a negative test.

mean level of ALT in the HGV RNA-positive patients (54 ± 2.6 U/l) was also significantly greater than in the HGV RNA-negative patients (15.2 ± 1.2 U/l, $p < 0.05$). There was no significant difference between the HGV RNA-positive and HGV RNA-negative patients regarding age, sex, serum anti-HCV and HBsAg.

To determine genotype distribution, the 9 HGV-positive samples were subjected to PCR with genotype-specific primers. Mix A buffer allowed for the specific detection of PCR products for types 1 and 3, and mix B for types 2 and 4, respectively. All 9 HGV-positive samples were identified as type 2 HGV based on PCR with the genotype-specific primers (fig. 1). The specificity of the results obtained by the PCR genotyping system was confirmed by sequencing analysis. The sequence analysis of 5' UTR of the 3 HGV isolates showed that all 3 belonged to subgroup 2a (fig. 2). Table 3 shows clinical, serological, and virological features of the 9 HD patients who were infected with HGV.

Fig. 2. Phylogenetic tree of three HGV isolates from East Anatolia, Turkey, generated by comparison of a 104-bp sequence in the 5' UTR of HGV. This tree was created using the neighbor-joining method in the Treecon (1.3b) program.

Discussion

It is known that HD patients are at increased risk for HGV infection [14, 19–21]. To the best of our knowledge, our study is the third report evaluating the prevalence of serum HGV RNA in HD patients in Turkey. Two previous studies [22, 23] found the prevalence of HGV RNA in HD patients to be 34.6 and 25%, respectively. In the present study, the prevalence of HGV RNA infection in patients was 10.2% compared to 3–57% [9, 19] in previous studies.

HGV and HCV belong to the same family of flaviviridae. The clinical characteristics and risk factors of HGV

Table 3. Characteristics of 9 HD patients who were infected with HGV

Patient	Age years/sex	Duration of HD, weeks	ALT U/l	Anti- HCV ¹	HGV genotype
1	58/F	3	44	–	2
2	21/F	104	25	–	2
3	39/F	468	33	+	2
4	69/M	3	11	–	2
5	55/F	208	12	–	2
6	25/M	52	142	–	2
7	30/M	360	188	+	2
8	81/F	156	11	–	2
9	63/F	260	20	–	2

¹ A plus sign denotes a positive test, and a minus sign a negative test.

infection may be similar to those of HCV infection. In our study, anti-HCV seropositivity was less common and was detected in only 6 patients (6.7%), including 2 who were also infected with HGV. Thus, HD patients are at risk for both HGV and HCV infections, and blood transfusion may be the route of transmission of the two viruses in these patients. It is possible that our study failed to show any relationship between HGV infection and inflammation of the liver because of the limited number of patients in our study and because, moreover, 1 patient who had elevated ALT level (188 U/l) was also infected with HCV. HGV is transmitted mainly by blood transfusion. In the current study, all transmission routes of HGV infection had not been clearly identified because all patients received blood transfusion.

Indeed, blood transfusion is the best-known transmission route of HGV RNA infection and all of our patients have a history of blood transfusion. In our study, the prevalence of HGV RNA (10.2%) was higher than anti-HCV seropositivity, probably because blood for transfusion had been serologically tested for anti-HCV since 1996 in Turkey. The 2 patients infected with HCV as well as HGV had been undergoing maintenance HD for a long period of time (468 and 360 weeks). We did not find any patient co-infected with HBV and HGV, probably because blood for transfusion had been examined for HBV, and HD patients who were negative for HBsAg were vaccinated against HBV.

It is known that the prevalence of HGV RNA is significantly correlated with the duration of HD [9, 24]. In

the current study, the mean duration of HD in the HGV RNA-positive patients (168.2 ± 50.1 weeks) was significantly longer than in the HGV RNA-negative patients (79.9 ± 15.9 weeks, $p < 0.05$) similar to other published reports [9, 23]. The mean level of ALT in the HGV RNA-positive patients (54.0 ± 21.6 U/l) was also significantly higher than in the HGV RNA-negative patients (15.2 ± 1.24 U/l, $p < 0.05$) in contrast to other published reports [20, 21], probably because there is no association between elevated levels of ALT and HGV RNA positivity.

Genotyping studies of HGV have been mainly conducted in industrialized [17] countries, hence it is not possible to determine the worldwide distribution of HGV. However, in Turkey, HGV genotyping done among renal

transplant recipients in Izmir, West Anatolia [25], showed that the dominant genotype was type 2 as observed in this study in East Anatolia, Turkey. However, molecular characterization of a larger number of HGV isolates may reveal further genotypes in Turkey.

Conclusion

HD patients are at increased risk of HGV infection in East Anatolia, Turkey. Anti-HCV seropositivity was lower in HGV RNA-positive patients than expected. Moreover, there was no co-infection with HBV. All the HGV isolates belonged to genotype 2.

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