HEPATITIS C VIRUS GENOTYPES AND HEPATITIS G VIRUS IN HEMODIALYSIS PATIENTS FROM SYRIA: IDENTIFICATION OF TWO NOVEL HEPATITIS C VIRUS SUBTYPES

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Abstract. High prevalence of hepatitis C (HCV) and hepatitis G (HGV) viruses has been reported among hemodialysis patients with substantial heterogeneity of HCV genotypes throughout the world. We studied HCV prevalence, clinical significance, genotype distribution, and HGV coinfection in hemodialysis patients from Syria. Ninety (75%) of 120 screened patients were HCV antibody positive. Forty-nine (87.5%) of 56 HCV antibody-positive patients had HCV RNA detected by the polymerase chain reaction. The HCV genotyping was possible in 37 of 49 patients (76%). The HCV genotype distribution was genotype 1a, seven (19%); genotype 1b, 10 (27%); genotype 4a, 11 (30%); unmatched sequences, nine (24%). Phylogenetic analysis of unmatched sequences indicated that they represent two distinct and novel subtypes of HCV genotype 4. Hepatitis G virus RNA was detected in 29 (59%) of the HCV RNA-positive patients. No differences were identified between patients infected with HCV alone and those coinfected with HGV. These data demonstrate that HCV infection is common in this population with a genotype distribution predominantly made up of types 1 and 4. Coinfection with HGV had no effect on the outcome of HCV infection.

MATERIALS AND METHODS

Samples and data collection. One hundred twenty patients undergoing regular hemodialysis for renal failure at a government-funded dialysis unit in Damascus, Syria were screened for HCV by a second-generation ELISA antibody assay (Boehringer, Mannheim, Germany or Abbott Laboratories, Delkenheim, Germany). Fifty-six HCV antibody-positive patients agreed to participate in the study. They provided a complete medical history and underwent a complete physical examination by one of the four investigators from Damascus University. Serum samples were collected and transported to the United States on dry ice for further analysis. Clinical data were gathered from each patient including demographic information and laboratory values such as aminotransferase, albumin, and total bilirubin levels. No liver biopsy specimens were available from any of the patients. Since the exact time of infection with HCV or HGV was not known, we used the time of first dialysis as an indicator of the duration of infection.

Sterilization of dialysis equipment was done by infusion of 200–300 ml of 5% sodium hypochlorite into the dialyzer followed by an equal volume of distilled water. Formalin (15%) was also used as an additional sterilization procedure on weekly basis a few months prior to the initiation of the study. Health care workers had been instructed and were expected to follow universal precautions at all times, but no mechanism to monitor compliance was identified. Informed consent was obtained from each patient prior to proceeding with the obtaining of blood. The study was approved by the Institutional Review Board of the Mayo Clinic.

Detection and genotyping of HCV RNA. Reverse transcription and PCR amplification. Direct nucleic acid se-
sequencing of the viral genome was used to distinguish HCV genotypes and subtypes. The HCV RNA was extracted from 100-μl aliquots of serum following the addition of 1 ml of RNAzol B solution (2 mol/L guanidinium thiocyanate, 12.5 M sodium citrate, 0.25% N-lauroylsarcosine, 0.05 mol/L 2-mercaptoethanol, 100 mmol/L sodium acetate, and 50% water-saturated phenol (Leedo Laboratories, Houston, TX). After addition of 100 μl of chloroform, samples were held at 4°C for 5 min, centrifuged for 15 min at 12,000 × g, and the aqueous phase was extracted. Total RNA was precipitated by the addition of an equal volume of isopropanol, 40 μg of glycogen, and incubation at 4°C for 45 min. The RNA pellet was recovered by centrifugation at 12,000 × g, washed in 1 ml of 70% ethanol, dried briefly, and resuspended in 25 μl of RNase-free water containing 40 units of recombinant RNAsin (Promega, Madison, WI).

For the purpose of detection, HCV RNA was reverse transcribed, and then amplified by PCR with primers specific for the 5′ untranslated region of the viral genome as previously described.17 For genotyping, the reverse transcription reaction was performed in a 20-μl reaction mixture that contained 1× reverse transcriptase buffer (100 mM Tris-HCl, 500 mM KCl, 1% Triton X-100, pH 8.6; Promega), 15 units of avian myeloblastosis virus reverse transcriptase (Promega), 20 units of recombinant RNAsin (Promega), 1 mM dNTPs, 3.0 mM MgCl₂, and 2.5 mM antisense primer (5′-CCGGGAATTCCTGGTCATAGCCTC TG TGAA-3′) for the nonstructural protein 5 (NS5) region or antisense primer (5′-TAC/TCCCATGAGGTCGGCA/GAAGC-3′) for the core region. Reactions took place at 42°C for 5 min, followed by 99°C for 5 min and 5°C for 5 min. The PCR amplification was accomplished by the addition of a second mixture containing 0.8× PCR buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3; Perkin Elmer, Norwalk, CT), 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), 200 mM dUTP, 10% (v/v) glycerol, 1.25 mM MgCl₂, and 0.5 mM sense primer (5′-TGGGATCCCTGGATATCCGCCTGCTCGTTGTA-3′) for the NS5 region or sense primer (5′-TGCTGTAGGCTTGTGGATC3′) for the core region, in a volume of 80 μl. The concentrations are based on final reaction volume of 100 μl. Amplifications were carried out in a DNA Thermal Cycler 480 (Perkin Elmer) as follows: 94°C for 4 min, then 50 cycles of 94°C for 1 min and 58°C for 1 min, followed by a final extension at 72°C for 5 min. Primers used in the core region are modified primers previously published by Mellor and others.18

The PCR reactions were analyzed by gel electrophoresis on a 2% Seakem agarose gel (FMC Bioproducts, Rockland, ME). To avoid possible contamination with exogenous sequences during extraction or amplification, and nucleic acid extraction, amplification and detection steps were performed in separate laboratories. Negative and positive controls were extracted, reverse transcribed, and amplified in each batch of samples tested by PCR. False-positive results were not obtained with the negative controls.

Sequencing and genotyping. The PCR products were puriﬁed prior to sequencing by using a direct column puriﬁcation method (QIAGEN PCR puriﬁcation kit; QIAGEN, Chatsworth, CA). Automated sequencing was performed in an ABI 373A or 377 (Applied Biosystems, Hercules, CA). The PCR products were sequenced in both directions using either the same primers used for the PCR or additional primers as in the core region with a sense primer (5′-AGGTCTCTGTAACCGTGACATG-3′) or an antisense primer (5′-CAC/TGTA/GAGGTTATCGATGAC-3′).

A contiguous sequence was constructed for the nucleotide sequences that were generated from each sample using MacVector 4.0 sequence analysis and Assemblylign sequence assembly software (Oxford Molecular Group Inc., Campbell, CA). The sequences were then aligned with previously reported sequences by using the Pileup software program included in the Wisconsin Package.19 Parsimony analysis by a heuristic algorithm was then performed with the Phylogenetic Analysis Using Parsimony (PAUP) version 3.1.1 computer program.20 Bootstrap replicates (n = 100) were constructed and a > 70% majority-rule consensus tree was established. Further analysis for conﬁrmation of branching order was performed with the Molecular Evolutionary Genetics Analysis (MEGA) version 1.01 computer program.21 A Jukes-Cantor distance measurement was established and neighbor joining analysis was performed with bootstrapping (n = 1,000). These methods allowed comparison of a 222-basepair (bp) and 308-bp portion of each viral genome homologous to nucleotide positions 7975 to 8196 (NS5) and 289 to 747 (core) in the prototype virus. A consensus nomenclature system for the classiﬁcation of different HCV genotypes was used for the purpose of this study.13

Extraction and detection of HGV RNA. The HGV RNA was extracted using the previously described RNAzol B Technique, and then reverse transcribed using a previously published primer 211R (5′-CGAATGAGTCAAGGAGCGGGGTAT-3′) or an antisense primer (5′-AGGTCTCTGTAACCGTGACATG-3′) for the 156-bp amplification product.14 Ampliﬁcation was accomplished by addition of a second mixture containing 0.8× PCR buffer II, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), 10% (v/v) glycerol, 1.75 mM MgCl₂, 190 mM dUTP, 10 mM digoxigenin dUTP (Boehringer Mannheim, Mannheim, Germany), 2 units of uracil-N-glycosylase (1 unit/μl; Epicenter Technologies, Madison, WI), and 0.5 mM sense primer 77F (5′-CTCTTTTGTGTAATGACCGGAGAT-3′).14 Amplifications were carried out in a DNA thermal cycler 480 as follows: 50°C for 10 min, 94°C for 4 min, then 50 cycles at 94°C for 1 min and 58°C for 1 min, followed by a final extension step at 72°C for 5 min. A 40-μl aliquot of each RT-PCR mixture was then tested using the PCR ELISA DIG Detection Kit (Boehringer Mannheim) and capture probe 152F (5′-TCGGTTACTGAGA GAAGC-3′) for detection of the 156-bp amplification product.14

Statistical analysis. The rank-sum and Kruskal-Wallis tests were used to compare continuous variables such as age between groups, and the Fisher exact test was used to assess associations. Because only one patient was infected with one of the two HCV genotypes, all tests of association between genotype and other factors are based on data that were collapsed into four groups: genotype 1a, genotype 1b, genotype 4a, and genotype 4* (patients infected with the two novel subtypes). The SAS statistical analysis package (SAS Institute, Cary, NC) was used for all calculations.

RESULTS

Detection of HCV and demographic data. Of 120 patients that were screened, 90 (75%) were HCV antibody pos-
HCV AND HGV GENOTYPES

573

FIGURE 1. Phylogenetic trees drawn from the parsimony analysis of nucleotide sequences by the heuristic method of the core region (tree A) and the nonstructural protein 5 (NS5) region (tree B). Sequences are indicated with their accession numbers and names if available. Numbers indicate branch length and percentages indicate bootstrap replicates (n = 100). Syr1 and Syr2 represent the novel subtypes from Syrian hemodialysis patients.

Detection of HGV RNA. Hepatitis G virus RNA was detected by a second-generation ELISA. No serologic confirmatory test such as an immunoblot assay was used. Hepatitis C virus RNA was detected in 49 of 56 samples (87.5%) that were transported to the United States. Samples arrived thawed due to a delay in the transportation process and therefore may underestimate the presence of viremia in this population. The 56 HCV antibody-positive patients included 33 males and 23 females. Ages ranged between 18 and 65 years (mean = 42 years). Six patients had a history of acute glomerulonephritis following acute pharyngitis, four had diabetic nephropathy, four had recurrent pyelonephritis, two had a history of renal calculi requiring surgical intervention, and four had chronic renal failure without a clear etiology (renal biopsies were not available). The majority (49 of 56, 87.5%) of these patients had received at least one transfusion of blood or blood products prior to HCV diagnosis. Of the seven patients with no history of transfusion, five had a surgical procedure performed at some time prior to HCV diagnosis, and two had no risk factor for HCV acquisition. There was no history of excessive alcohol intake or intravenous drug use in any of the study patients.

Compliance with universal precautions of the nursing staff and allied health workers was felt to be inadequate in the dialysis unit during the repeated brief visits of the investigators between January and June 1995.

Genotype distribution of HCV. Genotype determination of HCV was possible in 37 (76%) of the 49 HCV RNA-positive samples. The genotype distribution was as follows: HCV genotype 1a, seven (19%); HCV genotype 1b, 10 (27%); and HCV genotype 4a, 11 (30%). Nine samples (24%) were not classified into any of the known HCV subtypes but were closely related to HCV genotype 4. Eight of these uncategorized samples were closely related (97–100% similarity in a 222-bp sequence of the NS5 region) and one was distinct. There were no differences in the mean age (P = 0.11), aminotransferase level (P = 0.5), albumin (P = 0.48), total bilirubin (P = 0.28), or the estimated length of infection (P = 0.3) among patients infected with different HCV genotypes (1a, 1b, 4a, and 4*).

Identification of two novel HCV subtypes. Further analysis was carried out for the nine HCV isolates that were closely related but not identical to HCV genotype 4. Phylogenetic trees of the 222-bp fragment of the NS5 region between nucleotide positions 7975 and 8196 and the core region between nucleotide positions 1 and 573 were constructed (Figures 1 and 2). These phylogenetic trees allowed comparison of the genetic relatedness of our isolates, Syr1 and Syr2, to the published sequences related to HCV genotype 4 in NS5 (Eg19, Eg13, Gb215, L29607, Cam600, Gb809, Gb438, Gb549, and J6) and core region (N1, DK, Eg28, Eg15, Gb809, Cam600, Z4, Z1, Z8, N5, Z7, Z6, N2, Non, and D00944). In both regions, the calculated genetic distance indicated that Syr1 and Syr2 are indeed distinct subtypes of HCV genotype 4.

The novel sequences identified have been reported to the GenBank. They have been given the following accession numbers: Banklt 170 132 AF046823—Syr1/NS5, Banklt 170 153 AF046824—Syr2/NS5, Banklt 170 166 AF046825—Syr1/Core region, and Banklt 170 186 AF046826—Syr2/Core region.
detected in 29 (59%) of 49 samples. Again, there were no significant differences in HCV genotype distribution, mean aspartate aminotransferase, mean bilirubin, and mean albumin levels, or the duration of HCV infection among those infected with HGV and those who were not.

**DISCUSSION**

Exposure to blood or blood products by transfusion or intravenous drug abuse are the predominant modes of transmission of HCV.\(^{22-24}\) Clustering of HCV infection in patients with chronic renal failure receiving hemodialysis has also been described. The prevalence of HCV antibody positivity in dialysis patients ranges from about 1% in studies from Australia and New Zealand\(^{25,26}\) to approximately 50% in Vietnam, Taiwan, and Saudia Arabia.\(^{27-29}\) In this study, a prevalence rate of 75% was found in hemodialysis patients from Syria, which is one of the highest rates reported in the literature. It is not clear whether dialysis itself is a risk factor for HCV transmission or the high prevalence rate of HCV is a result of multiple blood transfusions in this population. Seven of our 56 patients (12.5%) never received blood or blood products, and at least in two of them, no other risk factor for HCV acquisition was identified, indicating that hemodialysis may be associated with an increased risk for HCV infection independent of blood transfusion. In one
study, a comparison was carried out between dialysis centers with high rates of HCV infection and those with low rates and found that the major difference between them was the adherence of the staff to universal precautions. This may also be a major contributing factor to the high rate of HCV infection at this dialysis center.

Since the exact time of HCV infection is not known in the majority of the study patients, we are not able to completely rule out the possibility that HCV was present prior to the time of dialysis and may have contributed to the deterioration of kidney function in these individuals. Recent evidence suggested that HCV infection may have an etiologic role in the pathogenesis of nephropathy, particularly membranoproliferative and membranous nephropathies.

It was thought that the deposition within the glomeruli of immune complexes containing HCV antigens, anti-HCV antibodies, and rheumatoid factors (anti-IgG antibodies) may be responsible for the renal disease in patients with chronic HCV infection. This hypothesis was supported by demonstrating immune complexes within the glomeruli in HCV-infected patients with glomerulonephritis using immunohistochemical methods. An alternative hypothesis is the production of autoantibodies to renal antigens in HCV-infected patients, which may lead to kidney damage. The latter hypothesis could be supported by the high prevalence of autoantibody formation in HCV patients.

Substantial sequence heterogeneity among patients with chronic HCV infection has been reported and was classified into multiple genotypes and subtypes. The distribution of HCV genotypes seems to vary from one geographic region to another around the world, which may have important implications in the development of a universally effective vaccine. In this study, we found that the HCV genotypes in hemodialysis patients from Syria are equally distributed between HCV genotype 1 and HCV genotype 4. Hepatitis C virus genotype 4 has been found mostly in patients from the Middle East. The greater diversity of sequences obtained from patients infected with genotype 4 in the Middle East, including the two new sequences reported in this study, might reflect endemic clustering of infection with HCV genotype 4 compared with other HCV genotypes.

Hepatitis G virus, an RNA virus, has been recently cloned and sequenced. The new virus appears to have similarity to the Flaviviridae family. The transmission of this virus through blood transfusion and by other parenteral routes of exposure, such as intravenous drug use, has been suggested. Approximately, 20% of the HCV-infected patients were reported to be coinfected with HGV. In hemodialysis patients from Japan, HGV was detected in 10% compared with 0.9% of the Japanese general population, a prevalence that is significantly lower than that in hemodialysis patients from Syria. Two of our HGV infected patients (4%) had no blood transfusion or needle exposure aside from hemodialysis. The high prevalence of HCV and HGV in hemodialysis patients, many of whom are multitransfused individuals, suggests similar modes of transmission.

In conclusion, we found a high prevalence of HCV and HGV in chronic renal failure patients undergoing hemodialysis in Syria. These rates may reflect less than optimal screening techniques of blood and blood products, poor sterilization of equipment used in dialysis, a high prevalence of these viruses in the general population, or a combination of these factors. The HCV genotypes 1 and 4 were predominant in the study population, and two novel subtypes were identified, which further expands the spectrum of genetic diversity of this virus.

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