Hepatitis C virus genotypes in chronic dialysis patients


Nephrology Department and Blood Transfusion Center, Hospital of Lecco, Department of Hygiene and Preventive Medicine, University of Milan, Italy

Abstract

Background. Hepatitis C virus (HCV) infection is highly prevalent in dialysis patients. To further characterize HCV infection in this patient population, we studied the distribution of viral genotypes in 55 patients undergoing chronic dialysis treatment with seropositivity for HCV markers.

Methods. Thirty-two of 55 (58%) patients showed HCV RNA in the serum using reverse transcription polymerase chain reaction (RT-PCR) in the 5'-untranslated region (UTR) of the viral genome. HCV genotyping was performed using biotinylated type-specific oligonucleotide probes after hybridization with amplified sample material.

Results. HCV subtype 2a was dominant (56%), followed by HCV subtype 1b (31%), type 3 (3%) and 4 (3%). There was no association between demographic or clinical features of this cohort and HCV genotype. Genotype dependence was observed for antibody response toward the NS4 (c 100–3 and 5–1–1) proteins, which was infrequent in genotype 2a carriers compared with genotype 1b (P = 0.004).

Conclusions. HCV subtype 2a was dominant in our cohort of anti-HCV-positive dialysis patients; there was no association between HCV genotypes and demographic or clinical features of patients; the absence of antibody response toward the NS4-related antigens was frequent in genotype 2a carriers and may serve to predict responses to interferon therapy. The relative homogeneity of the viral population in dialysis patients attending our unit suggests a nosocomial transmission of HCV in this clinical setting.

Key words: dialysis patients; HCV genotyping; NS4 proteins

Introduction

Scarcely information [1,2] exists about HCV genotyping in dialysis patients. Hepatitis C viruses (HCV) are a heterogeneous family of viruses made up of several major types. Currently five major genotypes have been characterized in detail, and each major type can, in turn, be differentiated into a number of subtypes. There are indications that different (sub)types of HCV may have different clinical relevance, such as the response to interferon treatment [3] and the risk of developing cirrhosis or hepatocellular carcinoma [4]. Furthermore, different HCV genotypes may evoke different serological responses [5]. Therefore the aims of this study were (a) to investigate the prevalence of HCV genotypes in a cohort of anti-HCV-positive dialysis patients, (b) to evaluate the relationship between HCV genotypes and demographic, clinical, or biochemical features of patients, and (c) to analyse the association between the distribution of HCV genotypes and antibody responses toward specific HCV proteins in chronic dialysis patients.

Subjects and methods

Patients

We included in the study a total of 55 patients who had been treated by dialysis for chronic renal failure. There were 26 males and 29 females with a median duration of dialysis treatment of 64 months (range 6–282). The mean age was 60.4 ± 13.1 years (range 27–85). We recruited all anti-HCV positive patients who underwent maintenance dialysis during February 1995 at a single dialysis unit in Lecco, northern Italy. Routine HD techniques were performed with 3- to 4-h treatments three times a week. Bicarbonate dialysis was used for 52 patients. Three patients were on continuous ambulatory peritoneal dialysis. The aetiology of renal failure was chronic glomerulonephritis (n = 15), polycystic kidney disease (n = 9), hypertension and nephrosclerosis (n = 9), diabetic nephropathy (n = 3), pyelonephritis (n = 11), end-stage renal disease of unknown aetiology and others (n = 8). Serum aspartate aminotransferase, alanine aminotransferase and gammaglutamyl transferase were performed using an automated multienzyme analyser. Hepatitis B surface antigen (HBSAg); hepatitis B e antigen (HBeAg); antibodies to hepatitis B surface (HBSAb), and core (HBCAb) antigens; and antibodies to the human immunodeficiency virus were measured by commercially available kits from Abbott Diagnostics. No patient admitted a history of intravenous drug abuse. No patient had detectable human immunodefici-
ency virus antibodies in our dialysis unit in spite of the fact that some had received several blood transfusions.

**Serological methods**

Screening tests. All patients were screened by second-generation ELISA test kit (Ortho; Ortho Diagnostic Systems, Raritan, New Jersey, USA) including antigens from structural (core) and non-structural (NS3 and NS4) regions of the HCV genome. All tests were carried out and interpreted strictly in accordance with the manufacturers' instructions. Initially reactive samples were re-tested by ELISA in duplicate.

**Recombinant immunoblot assays (RIBA)** Semi-quantitative confirmation of ELISA-positive samples was performed by using a second-generation immunoblot assay, (4-RIBA, Chiron Corporation and Ortho Diagnostic Systems) which uses four recombinant proteins. In this RIBA, antibodies against structural (c 22–3) and non-structural (c 33-c, c 100-3, and 5–1–1) recombinant antigens were determined with arbitrary rating of the intensity of the bands from 1+ to 4+. Interpretation of the RIBA HCV test results was carried out according to the directions outlined by the manufacturer [6].

**HCV genotyping** The HCV genotypes in the serum samples were determined using AmpliCoc HCV test (Roche Diagnostic System, Nutley, NJ) and a hybridization assay, called line probe assay (LiPA, Innogenetics, Zwijndrecht, Belgium), based on the reverse-hybridization principle. For the AmpliCoc HCV PCR kit, we followed the manufacturer's instructions [7]. Briefly RNA was extracted from 100 ml of serum with the lysis buffer containing guanidine thiocyanate and 2-mercaptoethanol in the presence of RNA carrier; RNA was then recovered by isopropanol precipitation. HCV RNA was reversely transcribed and amplified in a single-tube reaction using a ready-to-use master mix containing Tth DNA polymerase, Amperase, and primers KY 80 (sense 5’-GCA GAA AGC GTG TAG CCA TGG CGT 3’) and KY 78 (antisense 5’-CTC GCA AGC ACC CTA TCA GGC AGT 3’), which contain a biotin residue at the 5’ terminus to allow detection of amplification product. The conditions for RT-PCR were as follows: one cycle at 50°C for 2 min, 60°C for 30 min, 95°C for 1 min; two cycles at 95°C for 15 s, 60°C for 20 s; 38 cycles at 90°C for 15 s, 60°C for 20 s; one cycle at 60°C for 4 min. Samples were soaked at 72°C until use. Amplification products, after alkaline denaturation, were hybridized to probe KY 88 (5’ GTT GGG TCG CGA AAG GCA GAA AGC GTG TAG CCA TGG CGT 3’) and KY 74 (antisense 5’-CTC GCA AGC ACC CTA TCA GGC AGT 3’), immobilized in wells of microtitre plates. After the addition of avidin-peroxidase conjugate and chromogen substrate, the absorbance was read at 450 nm. Samples showing optical density (OD) values exceeding 0.4 were considered positive. Negative and positive controls were included in each run. LiPA technology allowed the determination of the five major genotypes and six subtypes [8]. Specific oligonucleotide probes immobilized as parallel lines on membrane strips were hybridized with amplified sample material (samples processed by AmpliCoc HCV PCR kit). During amplification, biotinylated primers were incorporated in the amplified DNA fragments. After hybridization, streptavidin labelled with alkaline phosphatase was added and became bound to any biotinylated hybrid previously formed. Incubation with NBT/BCIP chromogen resulted in a purple-brown precipitate. The reactivity of an amplified fragment with one or more lines on the strip allowed the recognition of the HCV genotype.

**Statistical analysis**

Data are expressed as mean±standard deviation. Non-parametric data are expressed as median with respective ranges. Group comparisons were made by chi-square test with Yates' correction or Fisher's exact test (for dichotomous variables) and a Mann-Whitney test for non-parametric values. A P value less than 0.05 was accepted as significant. Statistical analysis was performed with the computer program Package Primer (by Stanton A. Glantz, 1989).

**Results**

Thirty-two of 55 patients (58%) showed detectable HCV RNA in the serum; 30 of 32 (94%) were typed by the procedure used. HCV subtype 2a (type III, according to Okamoto) was largely found (18/32 = 56%), genotype 1b (type II) was detected in 10 patients (10/32 = 31%), genotype 3 (type IV) was detected in one patient (1/32 = 3%), and genotype 4 (not classified by Okamoto) in one patient (1/32 = 3%). None of our patients showed mixed HCV populations.

The demographic and clinical features of anti-HCV positive and negative patients are shown in Table 1. There were no significant differences between patients infected by subtype 2a and 1b with regard to age, sex and median duration of dialysis. There were no differences between patients infected with HCV subtype 2a and 1b with regard to mean values of current AST (20.4±8 vs. 24.9±8, NS), ALT values (23.8±1 versus 29.6±1, NS) and median levels of gamma-glutamyl transferase (20 versus 47, NS).

There were no differences between HCV RNA positive and negative patients regarding mean values of current AST and ALT concentrations. A significant difference (P=0.03) was found between viraemic and non-viraemic patients with regard to the median values of current gamma-glutamyl transferase levels, 47 (5–149) and 17 (5–78) U/l respectively.

The majority (61%, 8 of 13) of patients with increased aminotransferase levels at some time during dialysis treatment was infected by HCV type 2a; 3 patients (23%, 3 of 13) harboured HCV subtype 1b, one patient showed HCV type 3 infection (8%, 1 of 13). Half (53%, 10 of 19) of the patients with normal aminotransferase levels during dialysis treatment were infected by HCV type 2a, whereas 37% (7 of 19) were

**Table 1. Demographic and clinical features of HCV RNA positive patients**

<table>
<thead>
<tr>
<th>HCV genotype</th>
<th>n</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Duration of dialysis treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>18</td>
<td>10/8</td>
<td>62.7±13</td>
<td>80 (7–210)</td>
</tr>
<tr>
<td>1b</td>
<td>10</td>
<td>5/5*</td>
<td>62.8±10**</td>
<td>49*** (13–228)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1,0</td>
<td>57</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0/1</td>
<td>63</td>
<td>139</td>
</tr>
</tbody>
</table>

*HCV genotype 1b vs. 2a, NS. **HCV genotype 1b vs. 2a, NS. ***HCV genotype 1b vs. 2a, NS.
infected by HCV type 1b. Only one of these patients harboured HCV type 4 (1 of 19; 5%). There was no significant difference in the distribution of HCV genotypes among patients with raised or normal amino-transferase levels during dialysis treatment.

The frequency of raised gamma-glutamyl transferase at some time during dialysis treatment was 67% (12/18) in HCV genotype 2a carriers and 70% (7/10) among patients infected by HCV genotype 1b. The difference was not statistically significant.

There was no significant difference between the prevalence rates of HBV markers in HCV subtype 2a and 1b carriers; 55% (10/18) in patients infected with HCV subtype 2a and 40% (4/10) in genotype 1b carriers (NS).

All 32 HCV-RNA positive patients were considered reactive according to the 4-RIBA results. The detection of antibodies to HCV proteins in relation to HCV genotypes is shown in Table 2. As for each antibody response toward the four HCV proteins, antibodies to c22–3 were found very frequently, irrespective of the HCV genotype. They were found in all patients with genotype 2a and 1b. Antibody to NS3 (c33-c) protein was detected in the majority (61%; 11 of 18) patients with genotype 2a and in all patients with HCV subtype 1b. The difference was statistically significant (P = 0.001). The prevalence of antibody to NS4 (c5–1–1) protein was low in patients with genotype 2a (3/18 = 17%) compared with those with genotype 1b (8/10 = 80%; P = 0.004). Antibody to NS4 (c100–3) protein was detected in 22% (4/18) of patients with genotype 2a and in 80% (8/10) of patients with genotype 1b (P = 0.001).

HCV antibody levels in 4-RIBA were investigated in relation to HCV genotypes (Table 3). High levels of antibodies to c22–3 were seen irrespective of the HCV genotype. Antibody to NS4 (c100–3 and 5–1–1) protein showed higher levels in patients with genotype 2a than in those with genotype 1b. The difference was statistically significant (P = 0.001). Antibody levels to NS3 (c33c) were significantly higher in patients with genotype 2a compared to patients with genotype 1b (P = 0.001).

We found no relationship between HCV genotypes and anti-HCV IgM core antibody.

### Table 2. Detection of HCV antibodies in 30 HCV RNA positive patients

<table>
<thead>
<tr>
<th>HCV genotype</th>
<th>No</th>
<th>No (%) of cases with anti-HCV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>anti-core (c22-3)</td>
</tr>
<tr>
<td>2a</td>
<td>18</td>
<td>18 (100)</td>
</tr>
<tr>
<td>1b</td>
<td>10</td>
<td>10 (100)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1 (100)</td>
</tr>
</tbody>
</table>
| 4            | 1  | 1 (100)  | —         | —        | —        | *P = 0.027 HCV genotype 1b vs 2a; **P = 0.004 HCV genotype 1b vs 2a; ***P = 0.01 HCV genotype 1b vs 2a.

### Table 3. Detection of antibodies to HCV core (c22-3) protein, HCV NS3 (c33c) protein, and HCV NS4 (5-1-1 and c100-3) proteins in relation to HCV genotypes in dialysis patients with chronic type infection

<table>
<thead>
<tr>
<th></th>
<th>Genotype 2a</th>
<th>Genotype 1b</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HCV core (c22-3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(–)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>1 (6%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>(+)</td>
<td>2 (11%)</td>
<td>3 (30%)</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>15 (83%)</td>
<td>7 (70%)</td>
<td></td>
</tr>
<tr>
<td>Anti-HCV NS3 (c33-c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(–)</td>
<td>7 (39%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>5 (28%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>1 (5%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>3 (17%)</td>
<td>3 (30%)</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>2 (11%)</td>
<td>7 (70%)</td>
<td></td>
</tr>
<tr>
<td>Anti-HCV NS4 (c100-3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(–)</td>
<td>15 (83%)</td>
<td>2 (20%)</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>2 (11%)</td>
<td>3 (30%)</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>1 (6%)</td>
<td>2 (20%)</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>0</td>
<td>3 (30%)</td>
<td></td>
</tr>
</tbody>
</table>

*HCV antibody levels were expressed as RIBA band intensity.

### Discussion

Several different methods of genotyping HCV have been proposed. Once the material is amplified (by RT-PCR or other amplifying technique), a number of approaches can be used such as sequencing of each amplimer [9], restriction fragment length polymorphism (RFLP) [10], hybridization assays like line probe assay [8], DNA enzyme immunoassay [11], and finally type-specific primer nested PCR [12]. To perform HCV genotyping in our patient group we used LiPA technology, which allows an easy and fast determination of HCV types and subtypes according to the nomenclature suggested by Simmonds et al. [13] and the consensus system recently proposed [14].

We observed no difference between HCV genotypes regarding the duration of dialysis treatment. Such finding is in contrast with a very recent report of Pol et al. [1], who suggested a changing pattern of HCV genotype prevalence over time in the case of post-transfusional contamination of dialysis patients. A partially different route of HCV contamination might explain these conflicting results as several anti-HCV-positive dialysis patients of our study were not transfused.

The demographic, clinical, and biochemical characteristics were similar for each genotype.

We observed that antibody responses toward specific HCV proteins depend on HCV genotypes. An overall lack of reactivity with NS4 (c100–3 and 5–1–1) antigens was observed in patients with HCV type 2a.
This finding agrees with previous observations reported by others [15–17] and is probably related to the considerable sequence diversity of the NS4 region among genotypes. Indeed, anti NS4 antibodies in sera from HCV type 2a-infected patients are unlikely to recognize the NS4 protein fragments used for 4-RIBA assay that are produced according to the sequence of the HCV prototype (HCV genotype 1a). Research to date has shown that genotype 1b was associated with a poor response to interferon therapy; thus, nephrologists should keep in mind the presence and the levels of antibody to NS4 related antigens because they may serve to predict responses to interferon treatment.

In contrast with the results for the antibody to NS4 proteins, high levels of antibodies to core protein were found in the majority of patients irrespective of the HCV genotype, which suggests that the high antigenicity of core region of the prototype HCV is conserved among different HCV genotypes. This offers further evidence that antibodies to core protein can be used as very sensitive markers of HCV infection [18].

We found a statistically significant difference between patients with genotype 2a and 1b concerning antibody to NS3 antibodies. However, they were present in all patients infected with HCV subtype 1b and in the majority (61%) of patients infected with HCV subtype 2a.

The results shown in Tables 2 and 3 were confirmed when end-stage renal disease patients attending our unit (chronic dialysis patients and kidney graft recipients) were evaluated together [19].

The distribution of HCV subtypes in our dialysis patients is consistent with the results we found in kidney transplant recipients attending our unit [19]. On the other hand, these results differ markedly from that found in community-acquired HCV infection of northern and central Italy. In these latter cases genotype 1b was the most prevalent, followed by genotype 2b. Moreover, the distribution of HCV subtypes in our patients is different from that found in haemophiliacs of northern Italy who had been treated with commercial coagulation factor concentrates mostly, if not solely, imported from the United States. In these cases, genotype 1a was largely dominant, followed by genotype 2b. This result in Italian haemophiliacs reflects HCV genotype distribution in the United States as the consequence of the fact that the concentrates used by these patients were largely prepared from American blood donors.

Finally, in the current study we observed that HCV subtypes 2a and 1b accounted for the majority of circulating HCV viruses in dialysis patients attending our unit. The relative homogeneity of the viral population in dialysis patients attending our unit suggests a nosocomial transmission of HCV in the dialysis setting.

In conclusion, HCV subtype 2a was the most prevalent, followed by 1b, in our cohort of infected renal dialysis patients; there was no relationship between HCV subtypes and demographic or clinical features of patients; HCV genotypes have different antibody responses toward specific HCV antigens, and this may have implications for interferon therapy. The relative homogeneity of the viral population in our dialysis patients suggests a nosocomial transmission of HCV in the dialysis setting.

References


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