Molecular epidemiology of a hepatitis C virus outbreak in a haemodialysis unit

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Abstract

Background. Haemodialysis patients are at high risk of infection by hepatitis C virus. The aim of this study was to investigate a hepatitis C virus outbreak which occurred in a haemodialysis unit, using epidemiological and molecular methods.

Methods. Five seroconversions to hepatitis C virus antibody (anti-HCV) were observed over a 6 month period and these were added to the four previously recorded anti-HCV-positive patients. All nine patients involved in the outbreak were tested for HCV RNA by reverse transcription-polymerase chain reaction and hepatitis C genotype determination was accomplished by a reverse hybridization assay. Furthermore, part of the NS5 region of hepatitis C genome (nucleotide positions 7904–8304) was amplified and sequenced in all HCV RNA-positive patients. Then, phylogenetic analysis of the nucleotide sequences obtained was carried out in order to investigate any possible epidemiological linkage among patients. Detailed epidemiological records were also available for all haemodialysis patients.

Results. Samples from all five incident cases and three out of four prevalent HCV infections were found positive for HCV RNA. HCV genotyping studies revealed that all incident cases were classified as 4c/d, whereas one and two prevalent cases were 1a and 4c/d respectively. Sequence comparisons and phylogenetic tree analysis revealed that six of the patients harboured very similar strains and clustered together, including all incident and one prevalent case, which was implicated as index case. Further epidemiological analysis was consistent with patient to patient transmission.

Conclusions. Molecular and epidemiological analysis suggested that horizontal nosocomial patient to patient transmission was the most likely explanation for the virus spread within the haemodialysis unit under study.

Key words: genotyping, haemodialysis, hepatitis C virus, nosocomial infection, nucleic acid sequencing, phylogenetic analysis

Introduction

Haemodialysis patients are at high risk of acquiring hepatitis C infection since the prevalence of anti-HCV in this group is exceptionally high. Initial studies carried out with first generation enzyme-linked immunosorbent assays reported an HCV seroprevalence ranging from 2 to 47% [1–9]. With the introduction of second generation assays an almost two-fold increase in the prevalence of anti-HCV has been demonstrated [10–13].

A number of factors account for the increased risk of HCV infection in haemodialysis patients. These include blood transfusions [4,11,14–15] and duration of haemodialysis. Moreover, the presence of anti-HCV has been often documented in non-transfused haemodialysis patients suggestive of nosocomial transmission of HCV [2,5,7,8,16–20]. However, studies using molecular epidemiology methods provided conflicting results [21–26] and it has been postulated that in addition to patient to patient transmission, other recognized (blood products such as albumin, immunoglobulins) or unrecognized routes may be involved [25].

In the present study we describe an outbreak of
HCV infection in a haemodialysis unit. Molecular analysis of viral strains in association with epidemiological data reconstructed the plausible routes of transmission and provided evidence for a patient to patient nosocomial infection.

Subjects and methods

Haemodialysis procedure

At the time of the outbreak, 40 patients were regularly dialysed, on one of the two shifts per day (either morning or evening shift), 3 times per week on either a Monday-Wednesday-Friday or a Tuesday-Thursday-Saturday schedule. Thus, every dialysis machine was used by two persons per day. Patients were normally dialysed on the same shift and on the same console although exceptions could occur and these were recorded. The dialysis unit employed one large room with 10 dialysis machines (consoles no. 1–10) and a small room with one dialysis machine (console no. 11). Consoles no. 1–9 were used by all anti-HCV-positive and -negative patients, who were not separated, and shared the same dialysis machines. One dialysis machine was dedicated to patients in critical condition (console no. 10) and another machine in the small room (console no. 11) to HBsAg-positive patients (Figure 1). Nurses worked in two shifts per day and had to take care of three patients per shift, but they could also move from patient to patient if needed.

Haemodialysis was carried out using Gambro dialysis machines (Ab, Sweden, AK-10) and bicarbonate as dialysate. All patients were receiving heparin and dialysers were never reused. Machines were heat disinfected between treatments and chemically every month. AK-10 machines do not allow the dialysis procedure to start if the disinfection cycle has not been completed.

Data collection

The renal dialysis unit started its function in the beginning of 1989. Screening of anti-HCV antibodies and monitoring of alanine aminotransferase (ALT) was part of the haemodialysis unit routine since December 1989. By May 1993 an intensive serological follow-up study was initiated in all haemodialysed patients as part of the Multicenter Haemodialysis Cohort Study on Viral Hepatitis. Blood samples were collected every 2 weeks from anti-HCV-negative patients and every 4 weeks from anti-HCV-positive ones. Blood was centrifuged within 2 h from collection to achieve optimal conditions for HCV RNA detection. ALT levels were recorded and multiple serum aliquots from each bleed were kept at −70°C until further use.

Medical records were kept constantly for all patients as part of the haemodialysis unit routine. These records included data such as symptoms of hepatitis, history of liver enzyme abnormalities, medication history, past medical history and history of transfusions. Furthermore, the dialysis schedule (day and shift), seating arrangement (Figure 1), bleeding episodes, staff-patient assignments, work schedules, dialysis equipment maintenance as well as infection control practices were all recorded. Demographic and risk factors data including information on intravenous drug use, household contact with any hepatitis case, occupational exposure to human blood and temporary dialysis in other centres, were also available.

Hepatitis C serology

Routine detection of anti-HCV begun in December 1989, when the first generation enzyme immunoassay (ELISA) became available. A second generation immunoassay was adopted in June 1991 (Abbott’s Labs, Wiesbaden, Germany). All anti-HCV positive patients included in the study were confirmed by a second or third generation recombinant immunoblot assay (Riba-2; HCV-2.0 SIA or Riba-3; HCV-3.0 SIA, Chiron Corp., Emeryville, CA, USA).

Hepatitis C virus RNA quantification, extraction, amplification and genotyping

The detection of HCV RNA was carried out with the Amplicor HCV Test (Roche Diagnostic Systems Inc., Branchburg, NJ, USA) [27]. For the quantification of HCV RNA Quantiplex™ HCV RNA 2.0 Assay (bDNA), (Chiron Dignostics), was used. The determination of HCV genotypes was done on amplicons (generated by the Amplicor HCV Test), using a commercially available assay (Inno-Lipa HCV II, Innogenetics N.V, Zwijndrecht, Belgium). The last is a reverse amplification assay based on the hybridization of the
amplified sequence from the 5'-non coding region of HCV with oligonucleotide probes immobilized as parallel lines on membrane strips [28]. The probes correspond to the sequence of the six more common HCV genotypes and their subtypes. The nomenclature system proposed by Simmonds et al. was used [29].

To obtain sequences from the NS5 region of the viral genome, HCV RNA extraction, reverse transcription and amplification were done as follows: RNA was extracted from 100 µl of serum or plasma using a mono-phasic solution of phenol and guanidine isothiocyanate (Trizol™ LS Reagent, Gibco BRL, Grand Island, NY, USA), according to manufacturer's instructions. The final product was dissolved in 20 µl of RNase-free water. Reverse transcription was done using the Gene Amp RNA PCR Kit (Perkin Elmer Cetus, Branchburg, NJ, USA), in a total volume of 20 µl with 50 units of MuLV reverse transcriptase and random hexamers. The PCR reaction was carried out under standard conditions,
using 2.5 units of Taq polymerase (AmpliTaq, Perkin Elmer Cetus, Branchburg, NJ, USA), in a total volume of 100 μl on a DNA Thermal Cycler (Perkin Elmer Cetus 2400). The thermal profile of the reaction was as follows: initial denaturation at 94 °C for 2 min; 40 cycles with denaturation at 94 °C for 15 s, annealing at 45 °C for 30 s and polymerization at 72 °C for 30 s. In the PCR reaction and for the specific amplification of NS5 sequences, primers thought to be highly conserved amongst different variants of HCV were used [30]. The sequence of PCR primers were 243: 5’-TGGG GATCCCCGTAATGATACCCGCTGCTTTGGA-3’ (sense) and 242: 5’-GGCGGAA TTCCTGCTCATAGCCCTCCGTGA-3’ (anti-sense). The PCR product is a 400 bp fragment between HCV RNA nucleotide positions 7904 and 8304 based on the numbering system of Choo et al. [31,32]. The amplified products were electrophoresed through a 1.7% agarose gel and visualized by ethidium bromide staining. It should be noted that PCR reactions were carried out under strict conditions [33], in order to avoid contamination and false positive results. Furthermore, in every PCR run two reagent controls, two positive and two negative samples were also included.

Sequencing and phylogenetic analysis of viral strains

Amplified DNA was purified by the GeniePrep DNA isolation kit and directly subjected to double strand sequencing with dye-labelled dideoxy terminators. The ABI PRISM 377 automated sequencer (Applied Biosystems) was used for electrophoresis and data collection.

DNA sequences were edited and assembled using Sequencer software (Macintosh release 3.0). Clustal V was used for multiple sequence alignment of DNA sequences under default conditions [34]. Reference sequences for the various subtypes were obtained from the GenBank database.

Phylogenetic analysis of NS5 sequences was carried out in order to confirm the genotyping performed by Inno-Lipa and in addition, to investigate any possible epidemiological linkage among HCV strains from the haemodialysis patients. All the NS5 sequences were compared with each other and with selected HCV sequences of genotypes 1, 2, 3 and 4 from GenBank. Phylogenetic tree analysis was done by the neighbour-joining method using Kimura’s two parameter correction, as implemented in the PHYLIP package [35]. To further confirm the reliability of the phylogenetic tree, BOOTSTRAPPING (1000 replicates) was accomplished.

Results

Six patients who had been on haemodialysis were found positive for anti-HCV with 2nd generation HCV assays (June 1991). During 1992 three of these died, and one other patient seroconverted to anti-HCV (July 1992). No further seroconversions were observed until an outbreak of HCV infection was recorded between May and October 1993, when five new seroconversions to anti-HCV were noted (Table 1). At that time, the prevalence of anti-HCV in the haemodialysis unit was found to be 22.5%, which is much higher than the one reported for the general population (1.0% in our region). After October 1993, no more seroconversions occurred till the end of follow-up (October 1997).

A more intensive follow-up started in May 1993 with weekly or biweekly collection of samples when all patients were tested for HCV RNA and ALT level. At that time, eight patients of the haemodialysis unit were positive for HCV RNA. Three of them were prevalent cases of hepatitis C documented by anti-HCV antibodies, whereas the five were incident cases that seroconverted to anti-HCV between May and October 1993. All the other patients of the unit were negative for HCV RNA and did not develop anti-HCV antibodies until the end of the study. The five new cases of hepatitis C concerned patients who had never been transfused or had no transfusions of blood or blood products in the 14 months preceding seroconversion. Moreover, none of them had any other identifiable risk factor for HCV infection. It should be also noted that all members of the staff were regularly checked for HCV antibodies and were repeatedly found negative until the end of the study. The seroconverters had no obvious clinical symptoms and in two of them (HD 813 and HD 816) no ALT elevation was observed during the whole study period.

Genotyping using primers from the 5’-non-coding region (Inno-Lipa) revealed that seven out of the eight HCV RNA-positive patients belonged to subtype 4c/d (discrimination between subtypes 4c and 4d with Inno-Lipa method is not possible), including all the new seroconversions, whereas one patient was genotyped 1a (Table 1).

Sequences in the NS5 region of the viral genome were obtained with primers 242, 243 for all the HCV RNA positive patients of the study. In all cases, the first available serum sample with documented viraemia for hepatitis C was selected for sequence analysis (no serum samples were available before May 1993). Two other HCV RNA-positive patients, completely unrelated to the haemodialysis unit (Co1 and Co2), which were classified as 1b and 4 respectively by Inno-Lipa, were used as controls.

Phylogenetic tree analysis confirmed the HCV genotyping results obtained by Inno-Lipa for all the patients and controls, except for patient HD 819 who did not fall within the 4c clade, although it clearly clusters with type 4 isolates, as indicated by high bootstrap values (99%). In addition, genotyping by sequencing the NS5 region for the second control patient (Co2) was more precise than Inno-Lipa since it was clustered with the 4c group.

Table 1. Date of the first anti-HCV positive sample, HCV RNA and genotype status

<table>
<thead>
<tr>
<th>Patient</th>
<th>anti-HCV (+)</th>
<th>HCV RNA</th>
<th>HCV genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD 808</td>
<td>12/89</td>
<td>+</td>
<td>1a</td>
</tr>
<tr>
<td>HD 824</td>
<td>12/89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HD 819</td>
<td>6/91</td>
<td>+</td>
<td>4c/d</td>
</tr>
<tr>
<td>HD 836</td>
<td>7/92</td>
<td>+</td>
<td>4c/d</td>
</tr>
<tr>
<td>HD 807</td>
<td>5/93</td>
<td>+</td>
<td>4c/d</td>
</tr>
<tr>
<td>HD 806</td>
<td>6/93</td>
<td>+</td>
<td>4c/d</td>
</tr>
<tr>
<td>HD 804</td>
<td>7/93</td>
<td>+</td>
<td>4c/d</td>
</tr>
<tr>
<td>HD 813</td>
<td>8/93</td>
<td>+</td>
<td>4c/d</td>
</tr>
<tr>
<td>HD 816</td>
<td>10/93</td>
<td>+</td>
<td>4c/d</td>
</tr>
</tbody>
</table>
The very close relation between the six haemodialysis patients (HD 836, HD 807, HD 806, HD 804, HD 813, HD 816) which formed a distinct cluster, as well as the high bootstrap values (1000 of 1000), clearly points towards a common source of infection. Four out of six patients (HD 804, HD 816, HD 836, HD 813) displayed identical nucleotide sequences, and the two remaining (HD 806, HD 807) had one nucleotide substitution each. Phylogenetic analysis also revealed that (i) patient HD 819 was not associated with the outbreak and (ii) the index case was HD 836 who seroconverted to anti-HCV in July 1992. It should be also noted that in May 1993 HD 836 presented HCV RNA levels $1.13 \times 10^6$ Equivalents/ml and had ALT levels 104 IU/ml.

Discussion

There is much evidence supporting the nosocomial transmission of HCV infection among the haemodialysis patients of this study: (i) the existence of five seroconversions, which took place over a 6 month period and was observed in non-transfused patients, (ii) the finding of a single hepatitis C virus genotype (type 4) in all the patients of the outbreak, (iii) the extremely high prevalence of genotype 4 among the anti-HCV positive patients of the haemodialysis unit (77.8%), a genotype which is relatively rare in Greece (5%, in chronic hepatitis C patients, unpublished data) and (iv) the close relationship of the NS5 amplified sequences between the six haemodialysis patients as shown by phylogenetic tree analysis.

That nosocomial transmission was implicated, became apparent when molecular epidemiological studies were undertaken which included sequencing of the NS5 region of the HCV genome. The NS5 region has been used before for analogous studies [25,36]. Its suitability for phylogenetic analysis relies on the fact that it is relatively well conserved and therefore sequence changes in that part of the HCV genome, are slow enough to preserve evidence of relatedness over a considerable period of time [36]. Although the hypervariable region (HVR) of the E2 gene of hepatitis C has been used by many investigators for molecular epidemiology studies [37–43], it is believed that a higher rate of sequence changes might hinder attempts to link contact to the source of infection, as has been shown with the analogous V3 region of the envelope gene of HIV [44,45] and might lead to under-recognition of clusters infected from a common source, especially when samples are taken several years after infection [46]. Moreover our efforts to amplify HVR in genotype 4 samples, from various transmission groups was unsuccessful (data not shown). A similar epidemiological problem, the high prevalence of hepatitis C genotype 4 virus in patients treated in the same haemodialysis unit, was studied by Sampietro et al. [22] using single strand confirmation polymorphism (SSCP) analysis of polymerase chain reaction products obtained from the 5'-untranslated region of the viral genome.

Based on genotyping by Inno-Lipa, the suspected index cases were HD 819 or HD 836 or both. Both were of genotype 4 and known carriers before the present outbreak (June 1991 and July 1992 for HD 819 and HD 836, respectively). Phylogenetic tree analysis of the NS5 region ruled out that HD 819 was the index case. On the contrary, the same phylogenetic tree analysis placed the six remaining patients (HD 836, HD 807, HD 806, HD 804, HD 813, HD 816) in the same cluster. This cluster included the five new cases and patient HD 836 who was anti-HCV positive since July 1992, thus strongly suggesting that the latter was the index case of the outbreak.

Looking back to the epidemiological data it was found out that (i) patient HD 836 was regularly dialysed in the same shift with patient HD 813 (every Tuesday-Thursday-Saturday evening) from September 1992 and during the whole of 1993, but never shared the same dialysis machine suggesting the horizontal mode of transmission as the most probable explanation and (ii) four out of the five patients who seroconverted between May and October 1993 (HD 804, HD 806, HD 807, HD 816) were dialysed in the same morning shift (every Monday, Wednesday and Friday) when patient HD 836 was not regularly served. Nevertheless, according to the phylogenetic analysis, the infection presumably passed from patient HD 836 to the morning shift. Careful review of epidemiological records revealed that although patient HD 836 was regularly treated in the evening shift D, he exceptionally attended the morning shift A (console no. 10) once on Monday February 1, 1993. While anti-HCV seroconversions started in May 1993, late seroconversion to anti-HCV is well documented among haemodialysis patients [47–49]. These patients can be viraemic and therefore able to transmit hepatitis C, but anti-HCV is not detected for a relatively longer period of time. Thus, it is possible that one or more patients of the morning shift were accidentally infected in February 1993 and, being viraemic, they passed the transmission to the rest of the patients of the morning shift during the following months (horizontal mode of transmission).

Patients HD 836 and HD 816 shared the same dialysis machine. However, a vertical route of transmission through the dialysis machines is a very remote probability since patient HD 836 used to be in the Tuesday-Thursday-Saturday evening shift, after which the dialysis machines were routinely carefully disinfected and dried. The unit was ending its operation around 20.00 h starting again next morning (Monday-Wednesday-Friday) around 08.00 h and it was in this shift that patient HD 816 was dialysed. It is also worth mentioning that AK-10 machines do not allow for the dialysis procedure to start if the disinfection cycle has not been completed; they drain utterly following disinfection and no dialysate recirculation takes place in any part of the hydraulic circuit.

Retrospective analysis of probable risk practices suggested that the vehicle of transmission was a nurse.
or a group of nurses who did not change gloves regularly when moving from patient to patient.

By December 1993, additional measures were adopted to prevent further spread of the virus: (i) all anti-HCV-positive patients were moved into one dialysis shift and (ii) frequent reminders to staff and patients for strict adherence to the universal precautions for the prevention of spread of viral diseases in the dialysis environment, as recommended by CDC, were applied. During the following 4 year period (January 1994–October 1997) no new seroconversion has been observed. Patient HD 836 was successfully transplanted (May 1994), HD 819 has been transferred to another unit (July 1994), patient HD 816 died from septicemia (February 1996) and HD 806 from cerebrovascular accident (June 1997). HCV prevalence in the unit at the end of the study period was 11.4% (5/44).

The molecular and epidemiological investigation of this outbreak, in addition to the majority of HCV outbreaks studied by molecular epidemiology methods [21–24,26], suggest that patient to patient transmission of the hepatitis C virus by second generation test in hemodialyzed patients. *Kidney Int* 1993; 43 [Suppl 41]: S149–S152. Additional measures were adopted to prevent further spread of the virus: (i) all anti-HCV-positive patients were moved into one dialysis shift and (ii) frequent reminders to staff and patients for strict adherence to the universal precautions for the prevention of spread of viral diseases in the dialysis environment, as recommended by CDC, were applied. During the following 4 year period (January 1994–October 1997) no new seroconversion has been observed. Patient HD 836 was successfully transplanted (May 1994), HD 819 has been transferred to another unit (July 1994), patient HD 816 died from septicemia (February 1996) and HD 806 from cerebrovascular accident (June 1997). HCV prevalence in the unit at the end of the study period was 11.4% (5/44).

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