Change in Hepatitis C Virus Genotype in Hemodialysis Patients After End-of-Treatment Response to Interferon Monotherapy—Relapse or Re-Infection?

Teresa C. Arrais,^{1*} Sonia Van Dooren,² Anne-Mieke Vandamme,² Christian Brechot,^{3,4,5} Francois Rimlinger,^{3,4,5} Antonio Eduardo Silva,¹ Renata M. Perez,¹ Maria Lucia G. Ferraz,¹ and Valerie Thiers^{3,4,5}

¹Federal University of São Paulo, UNIFESP, São Paulo, Brazil

²Rega Institute for Medical Research, KULeuven, Leuven, Belgium

³Department of Virology, Institut Pasteur, Paris, France

⁴INSERM U785, Villejuif, France

⁵University Paris-Sud, UMR-S 785, Villejuif, France

Hepatitis C virus (HCV) infection remains common among hemodialysis patients and its occurrence is related mainly to nosocomial spread. Although dialysis patients with HCV infection respond well to interferon-based therapy, relapse is frequent. This study aimed at a selected group of hemodialysis patients infected with HCV infection undergoing interferon therapy who achieved end-of-treatment virological response but became HCV-RNA positive again 6 months after end-of-treatment. It was evaluated whether de novo HCV-RNA positivity in these non-sustained responders occurred due to lack of clearance of HCV after the initial response to interferon-alpha (relapse) or due to re-infection with a new strain (re-infection). Genotyping by Inno-LiPA and by phylogenetic tree analysis using partial HCV-NS5B sequences at two evaluation points: pre-treatment (T0) and 6 months after end-of-treatment (T18). Non-sustained responders ($n = 15$) carried subtypes 1a (8 patients), 1b (4 patients), 3a (2 patients), and 4a (1 patient) before treatment. Identical subtypes were detected in 10 patients at T18. Five patients changed genotypes at T18, suggesting nosocomial re-infection. This study emphasizes the importance of epidemiologic measures to control the re-exposure of hemodialysis patients treated previously for HCV infection. **J. Med. Virol.** 80:80–86, 2008. © 2007 Wiley-Liss, Inc.

KEY WORDS: HCV; hemodialysis; nosocomial transmission; genotype; NS5B region

INTRODUCTION

Hepatitis C virus (HCV) chronic infection causes significant morbidity and mortality among patients undergoing hemodialysis. Epidemiological studies have clearly indicated that hemodialysis patients have a high risk of acquiring HCV infection [de Lamballerie et al., 1996; Olmer et al., 1997; Le Pogam et al., 1998; Kokubo et al., 2002]. Several risk factors have been identified for HCV infection in hemodialysis patients, which include the number of blood transfusions, the duration of hemodialysis and the prevalence of HCV infection in the dialysis unit [Pol et al., 2002]. Nosocomial transmission of HCV on dialysis unit is considered to be an important mode of transmission of the virus [Sanchez-Tapias, 1999].

HCV is classified into six major genotypes. Within the genotypes there are many subtypes, with varying geographic distributions and modes of transmission [Simmonds, 2004]. The HCV genotype is now recognized to be the strongest predictive variable regarding the

Abbreviations used: HCV; hepatitis C virus; IFN; interferon alpha; ALT; alanine aminotransferase; AST; aspartate aminotransferase.

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*Correspondence to: Teresa C. Arrais, Infectious Diseases, Virology Department, Room Ee 1767, Erasmus MC, Faculty Building's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands. E-mail: tarrais@uol.com.br

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response to interferon alpha (IFN) treatment. According to a recent meta-analysis, the IFN-monotherapy treatment of HCV-infected hemodialysis patients with genotype 1 is associated with a sustained virological response of 30.6% (95% CI: 20.9–48%) [Fabrizi et al., 2003b]. The associate use of ribavirin in this population has not been recommended because of frequent side-effect dose-dependent hemolytic anemia.

In this study, HCV genotype and subtype in a selected HCV-infected hemodialysis patient group of non-sustained responders before treatment and 6 months after end-of-treatment was retrospectively investigated to evaluate whether de novo HCV-RNA detection occurred due to lack of viral clearance after apparent initial response to IFN (relapse) or due to contamination with a new strain (re-infection).

MATERIALS AND METHODS

Patients

During the period from 1999 to 2002, HCV-infected hemodialysis patients with virological response during interferon therapy who presented de novo HCV-RNA detection 6 months after end of treatment (non-sustained response) were selected. The patients were referred from different dialysis units to the Gastroenterology Division at Federal University of São Paulo, Brazil. All patients fulfilled the following criteria: seronegativity for hepatitis B virus and human immunodeficiency virus infections and drug- and alcohol-free history. Patients received IFN at a dose of 3 MU three times weekly for one year and they were not exposed during the post-treatment follow-up to risk factors for HCV infection other than hemodialysis. The indications of treatment were chronic infection in 11 patients and acute infection in 4 of them.

Sera Samples

Sera samples were collected pre-treatment (T0), and 6 months after end-treatment (T18). All sera were collected prior to hemodialysis procedure, aliquoted immediately after blood collection and frozen at -20°C . Samples were subjected to alanine aminotransferase (ALT) determination by an automated kinetic method (normal value: 30 IU/L).

Diagnosis of HCV Infection

The diagnosis of HCV infection was based on the presence of anti-HCV antibodies by a third generation ELISA (IMX, Abbott Laboratories®, Chicago, IL). The third generation assay detects antibodies from three viral antigens (c22-3, c-200, and NS5) originating from four regions of the viral genome (core, NS3, NS4, and NS5). Acute HCV infection was defined as elevation of serum ALT followed by anti-HCV and HCV-RNA positivity in the sera of previous negative patients.

Criteria for Virological Response

Patients who were negative for HCV-RNA at the end of 12 months of therapy were classified as end-of-treatment virological responders [NIH, 2002]. All patients were considered to have virological non-sustained response once they had detectable serum HCV-RNA levels at 6 months after end-of-treatment.

RNA Extraction

HCV-RNA was extracted from 160 μl of serum by use of the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions.

Quantitative HCV-RNA Test

The AMPLICOR v1.0 assay was used according to the manufacturer's instructions (Roche Diagnostic Systems, Basel, Switzerland). The linearity of the assay range is 10^3 to 10^6 RNA copies/ml. Quantification analyses were done at T0 and T18.

Inno-LiPA II Testing

A second-generation line probe assay (Inno-LiPA II test; Innogenetics, Ghent, Belgium) was performed according to the manufacturer's recommendation, at evaluation points T0 and T18. In this procedure, labeled PCR products obtained from the 5'NTR are hybridized to immobilized oligonucleotide probes, which are specific for the six major types and can identify most subtypes.

PCR Amplification of HCV-NS5B Region

RNA was reverse transcribed by use of random hexamers primers (Applied Biosystems, Foster City, CA) and 200 U of SuperScript™ II RNase H⁻ Reverse Transcriptase (SSII RT; Invitrogen, Carlsbad, CA). A partial region of the HCV-NS5B gene was amplified from 8 μl of cDNA by nested reverse-transcription polymerase chain reaction forming a 323 base pairs product (amino acid position 8286→8609). This NS5B region was amplified using two sets of primers; for the first round (outer), NS5b-1S (5'-TATGAYACCCGCTGYTTT-GACTC-3') and NS5b-2AS (5'-GCNGARTAYCTVGTCA-TAGCCTC-3'); for the second round (inner), NS5b-3S (5'-CGCTGYTTTGACTCMACRGTCAC-3') and NS5b-4AS (5'-GTCATAGCCTCCGTGAAGACTC-3'). The thermocycler machine was set for one cycle of 15 min at 95°C , 1 min at 48°C , and 1 min at 72°C , followed by 25 cycles of 94°C for 10 sec, 52°C for 20 sec, and 72°C for 30 sec, followed by a final step of 10 min at 72°C . The second round nested amplification was set for 35 cycles of 94°C for 10 sec, 52°C for 20 sec, and 72°C for 30 sec, followed by a final step of 10 min at 72°C . Into the first-round PCR, 2.5 U of AmpliTaq Gold™ (Perkin Elmer, Waltham, MA) was added. Both first-round (30 μl) and second-round PCR (75.5 μl) contained 10 pmol each of each sense and antisense primer, 2.5 mM MgCl_2 (Perkin Elmer) and 1 mM dNTPs (Eurobio, Montpellier, France).

Sequence Analysis

Amplified PCR products were purified by use of the QIAquick PCR Purification Kit (Qiagen) and bidirectionally sequenced by use of the automate ABI PRISMA 377 DNA Sequencer (Applied Biosystems) using inner PCR primers. The sequencing arrays were performed according to the instructions of ABI PRISMA Dye Terminator Cycle Sequencing Ready Reaction Kit 3.0 (Applied Biosystems).

Genotyping by Phylogenetic Analysis

The sequences for NS5B region were aligned by use of Clustal_X, manually edited, and gap stripped [Thompson et al., 1997]. Using the MEGA program [Kumar et al., 2004], a phylogenetic tree was constructed by neighbor-joining tree method applied to the distance matrix obtained under the Kimura's two parameter and Tamura-Nei models [Tamura et al., 2004]. Tree was visualized with Mega and Treeview [Page, 1996]. To confirm the reliability of the phylogenetic trees, the bootstrap re-sampling test was performed 1,000 times. HCV sequences were identified as being significantly related if their bootstrap value was $\geq 75\%$ [Yves Van de Peer, 2003].

List of Reference Sequences

For genotype and subtype determination, the NS5B region was compared to sequences from blood-related HCV transmission already published in GeneBank/EMBL/DDBJ database. In order to obtain a complete phylogenetic tree reference sequences of the confirmed HCV subtypes [Simmonds et al., 2005] were included.

Nucleotide Sequence Accession Numbers

HCV-NS5B gene sequences described in this study have been submitted to GenBank and can be retrieved under accession numbers: DQ911561 to DQ911590.

Statistical Analysis

Data were expressed as mean $\pm$ SD. The 95% confidence intervals for proportion of subtypes frequencies were calculated using the binominal distribution. The proportions of high-ALT levels at T0 and T18 were compared using the McNemar test. The HCV-RNA load levels at T0 and T18 were compared using the Wilcoxon test. *P* values lower than 0.05 were considered significant.

RESULTS

Baseline Characteristics and HCV Viral Load

The clinical and laboratorial data of the patients are presented in Table I. All patients underwent blood transfusions. There were no significant differences in ALT levels within T0 and T18 time points.

HCV viremia was positive in all patients before antiviral treatment. Mean HCV-RNA concentration

TABLE I. Characteristics of the Patients

Age, years	44 $\pm$ 13
Gender: male/female	10/5
Diagnosis	Acute hepatitis: 27% Chronic hepatitis: 73%
Duration of hemodialysis, months	68 $\pm$ 43
ALT ^a >30 IU/L, n (%)	Pre-treatment: 13 (87%)* Post-treatment: 9 (60%)
Viral load, copies $\times 10^3$ /ml	Pre-treatment: 447 $\pm$ 211** Post-treatment: 460 $\pm$ 234

^aALT, alanine aminotransferase; normal reference ranges: 4–30 IU/L.

**P* = 0.12 pre-treatment versus post-treatment.

***P* = 0.29 pre-treatment versus post-treatment.

before treatment was 447 $\times 10^3$ copies/ml (range 137 $\times 10^3$ to 795 $\times 10^3$ copies/ml). At the T18, all patients were positive with mean HCV-RNA concentration of 460 $\times 10^3$ copies/ml (range 100 $\times 10^3$ to 827 $\times 10^3$ copies/ml). A significant difference in the HCV-RNA levels between pre-treatment and 6-months post-treatment values was not found, even in patients who changed genotypes/subtypes (*P* = 0.29).

Genotyping

All 30 sera samples from T0 and T18 tested for genotyping in this study were HCV-RNA positive by PCR. They were further genotyped with Inno-LiPA and by PCR amplification and submitted to sequencing and phylogenetic analysis of the HCV NS5B region. The distribution of HCV genotypes is presented in Table II. Ten of the 15 patients had identical genotypes and subtypes before and 6 months after end-of-treatment (67%: 95% CI: 44–90%). Five patients changed genotypes: they were initially 1b, 1b, 4a, 1b, 1a and changed to subtypes 3a, 1a, 1a, 2a, 2a, respectively. Among the

TABLE II. Results of HCV Genotyping by HCV-NS5B Region Amplification and Inno-LiPA

Patient	Inno-LiPA	NS5B sequences phylogenetic analysis
	T0/T18	T0/T18
1	1b/1b	1b/1b
2 ^a	1b/3a	1b/3a
3	1a/1a	1a/1a
4 ^b	1b/1b	1b/1a
5	1a/1a	1a/1a
6	3a/3a	3a/3a
7	1a/1a	1a/1a
8	1a/1a	1a/1a
9	3a/3a	3a/3a
10	1a/1a	1a/1a
11 ^a	4/1a	4a/1a
12	1a/1a	1a/1a
13	1a/1a	1a/1a
14 ^a	1b/2	1b/2a
15 ^a	1a/2	1a/2a

T0: pre-treatment; T18: 6 months after end-treatment.

^aPatients who changed genotypes.

^bPatient with discrepant test result.

patients who changed the genotype, three (P11, P14, P15) were referred from the same hemodialysis unit.

Phylogenetic Analysis

For genotype and subtype determination a phylogenetic tree construction was carried out including reference strains of confirmed HCV subtypes (Fig. 1). The 30 HCV strains from the 15 hemodialysis patients were distributed into subtypes: 1a 56% (17/30), 1b 17% (5/30), 3a 17% (5/30), 2a 7% (2/30), and 4a 3% (1/30). Two isolates from different patients that were grouped in HCV2a (P14T18 and P15T18) clustered together with a bootstrap support of 98%. Another two isolates belonging to subtype 1a (P11T18 and P15T0) also clustered closely together with a bootstrap support of 99%. All sequences from the 10 patients who rebounded with the same subtype were closely clustered with high bootstrap support.

DISCUSSION

Hemodialysis patients are at higher risk for HCV infection than the healthy population [Fabrizi et al., 2002; Poordad et al., 2004]. The Center for Diseases Control (CDC) reported a 8.9% nationwide prevalence of anti-HCV among hemodialysis patients in the United States in 2001 [MMWR, 2001]. A recent review also showed that Italy and France had a prevalence of HCV in dialysis patients of 20.5% and 10.4%, respectively [Prati, 2006]. In Brazil, the prevalence is even higher, ranging from 35.5% in the Southeast region to 45.5% in the Northern region [Moreira et al., 2005]. Extremely high prevalences of 94.7% have been reported from Saudi Arabia [Huraib et al., 1995] and from Moldavia with 75% [Covic et al., 1999]. One of the reasons of this prevalence variability can be explained by the inherent difficulties in evaluating HCV infection in hemodialysis patients. Liver function tests, such as AST and ALT, are normal in many cases, despite the viremia [Espinosa et al., 2002]. Results of false negative tests have been reported and detection of HCV viremia requires HCV-RNA testing, which is an expensive procedure [Fabrizi et al., 2005]. Samples for HCV-RNA testing in dialysis patients should be obtained prior to the hemodialysis procedure, and the use of heparin during dialysis sessions can interfere with the PCR technique [Degos et al., 2001; Tanaka et al., 2003]. In addition, the hemodialysis procedure lowers HCV-RNA levels by adsorption of HCV-RNA onto the inner surface of dialyzers and destruction of viral particles by the hydraulic pressure exerted by the blood for dialysis [Fabrizi et al., 2003a].

There is a controversial debate in the literature concerning strategies to control the transmission of HCV infection in hemodialysis units. Patient isolation, use of dedicated machines and a ban on dialyzer reuse are some of the strategies that have been proposed [Salama et al., 2000; Sypsa et al., 2005]. However, the CDC does not recommend such strategies because, although isolation may protect uninfected patients, it

Fig. 1. Neighbor-joining tree obtained for the NS5B region of 30 samples from the hemodialysis patients analyzed in this study and 40 unrelated sequences. Nodes with bootstrap support higher than 75% are indicated. P11T18 and P15T0 (subtype 1a) and P14T18 and P15T18 (subtype 2a)—underlined—were found clustering together with high bootstrap support, suggesting nosocomial infection. (P, patient; T, time point.)

might also increase the risk of superinfection in patients originally infected with a single strain, leading to relaxation of universal prevention measures.

Nosocomial HCV transmission in hemodialysis centers have been documented before [Katsoulidou et al., 1999; Gonzalez-Candelas et al., 2003; Furusyo et al., 2004], but the magnitude of the problem is not known yet. This study investigated a population of hemodialysis patients treated apparently successfully for 1 year with INF, as judged by a negative HCV-RNA test at the end of treatment. The population consisted of 15 patients who became renewed HCV-RNA positive within 6 months after stopping treatment, and who had no risk factors in this period other than hemodialysis. It was investigated whether HCV-RNA became positive again due to lack of sustained response (relapse) or due to contamination with a new strain (re-infection) by comparing the genotype and nucleotide sequence before treatment and 6 months after stopping treatment. A change of genotype or subtype would support the hypothesis of nosocomial re-infection in this 6 months period.

Molecular biological techniques such as genotyping and phylogenetic analysis have previously been used as a tool for research or epidemiological investigations for tracing the source of HCV infection within individual hemodialysis units. As an example, two studies supported nosocomial transmission including possible patient-to-patient cross-infection using phylogenetic analysis of the viral genome [Ansaldi et al., 2003; Bracho et al., 2005]. Numerous tests can be used for HCV genotyping, the fastest one being the Inno-LiPA test. However, the 5'NTR region is not heterogeneous enough for use in determination of the subtype and cannot be used for differentiation of HCV subtypes 1a and 1b [Chen and Week, 2002]. Nucleic acid sequencing of the NS5 region is generally considered to be the golden standard and the Simmonds classification of HCV genotypes is based on this genomic region.

The patients in this research were genotyped by Inno-LiPA in the 5'NTR and by phylogenetic tree analysis using partial HCV NS5B sequences. A discrepant result between Inno-LiPA and NS5B sequencing was found in patient number 4. In this patient, the HCV subtype 1b infection was detected with Inno-LiPA at T0 and T18, while amplification of the NS5B region demonstrated a HCV hetero-subtypic change from HCV1b at T0 to HCV1a at T18. Inno-LiPA is considered a reliable technique for HCV genotyping, however, discrepancies between Inno-LiPA HCV genotype 1 subtypic discrimination, based on a few polymorphisms only, and phylogenetic analysis based on nucleotide sequences, have been reported before [Laperche et al., 2005]. Since phylogenetic analysis is the more accurate method, it was assumed here a failure of the Inno-LiPA subtyping and consider Patient 4 as having changed subtype. According to the combined results, five patients changed the genotypes/subtype, which is suggestive of re-infection. In the other 10 patients, the non-sustained response was caused by the same subtype, implying

either relapse or re-infection with the same genotype/subtype.

Considering the relapse hypothesis, the authors speculate that HCV viral load was not present in the serum at the time of testing because of either the treatment or the disappearance of HCV-RNA to extravascular sites [Radkowski et al., 2005]. Regarding the hypothesis of re-infection, the five patients could be infected with multiple HCV strains and one strain would prevail as the viremic virus. After the end of a successful treatment, one displaced strain would become predominant and influence the outcome of therapy [Schroter et al., 2003]. Although some studies have described superinfection in intravenous drug users [Herring et al., 2004] and hemodialysis patients [Qian et al., 2000], this event is uncommon [Lau et al., 1996] and probably can not explain the switch in genotype in these five patients. Nevertheless, for a more detailed analysis of multiple infections, sequencing of several clones is needed.

The epidemiological investigation showed that Patients 11, 14, and 15 had histories of sharing hemodialysis rooms on same days and shifts, which could suggested nosocomial infection transmitted by contaminated material or through medical staff. In addition, Patient 11 was infected with HCV genotype 4 that was found in another infected dialysis patient in whom the follow-up was lost. The phylogenetic analysis suggested a patient-to-patient transmission, which corroborates the epidemiological findings of nosocomial infection (Fig. 1). According to this analysis, patient 15 was probably the source of infection to Patient 11 and, because Patients 14 and 15 belonged to HCV genotype 2a, had probably the same source of infection.

Epidemiological and molecular studies have focused attention upon the prevalence of nosocomial transmission of HCV infection in hemodialysis patients [Hmaied et al., 2004; Carneiro et al., 2007]. This study, however, moved to a step further, with an emphasis on nosocomial infection in a selected group of hemodialysis patients previously treated with IFN who became HCV-RNA positive again after achieved virological response. Thus, among 15 patients with non-sustained response after apparently successful treatment, there were indications that at least three of them suffered a nosocomial re-infection within 6 months after stopping treatment, finding that has not been reported before among HCV-treated hemodialysis patients. The main practical implication of this observation is the clinical differentiation between non-sustained response to IFN therapy and nosocomial re-infection in HCV-infected hemodialysis patients. Because nosocomial re-infection implies a re-treatment and a burden to the patient, a more rigorous attention should be paid to the post-treatment follow-up in HCV-infected hemodialysis patients.

In conclusion, the nucleic acid-based techniques Inno-LiPA and sequencing of the NS5 region was used to evaluate whether de novo HCV-RNA detection in non-sustained responders HCV-infected hemodialysis patients occurs due to lack of sustained response to IFN

(relapse) or due to contamination with a new strain (re-infection). A substantial number of the hemodialysis patients in this study changed HCV genotype, suggesting a high incidence of nosocomial re-infection. Even though more studies are needed to properly evaluate the magnitude of the risk for nosocomial infection in hemodialysis centers, these research results suggest a possible alarming incidence of re-infection in successfully treated patients, which should be taken into account in the discussion on recommendations to control the transmission of HCV infection and post-treatment follow-up in hemodialysis units.

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