

Search for the presence of occult hepatitis C in patients with treatment-induced viral clearance using an ultrasensitive assay

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SUMMARY

Introduction Occult hepatitis C is defined by the presence of virus in the peripheral blood mononuclear cells (PBMCs) and/or liver cells, in the absence of serum viremia.

Objective To detect the persistence of occult hepatitis C in hemodialysis (HD) patients and patients without renal disease (non-renal) with treatment-induced clearance of hepatitis C virus (HCV) infection, using assays with a very low detection limit of viremia.

Methods A group of 13 HD patients and a group of 43 non-renal patients, with treatment-induced HCV infection clearance were investigated in the study. The HD patients were treated with pegylated interferon alpha (PEG-IFN- α) only, while the non-renal patients were treated with a combination therapy of PEG-IFN- α and ribavirin. Detection of a possible persistence of HCV RNA in the PBMCs and plasma samples was assessed by an ultrasensitive reverse transcription polymerase chain reaction (RT-PCR) assay (2 IU/ml).

Results HCV RNA was not detected in the PBMCs and plasma samples of HD patients and of non-renal patients, when assessed by the ultrasensitive RT-PCR assay.

Conclusion When a sensitive RT-PCR assay was applied, to determine if treatment induced clearance of HCV infection had been successful, occult hepatitis C could not be detected by an ultrasensitive assay, neither in HD nor in non-renal patients.

Keywords: hepatitis C virus; treatment, pegylated interferon α ; ribavirin; hemodialysis; mononuclear leukocytes

INTRODUCTION

Occult hepatitis C has been described as a different pathological entity than chronic hepatitis C. The presence of detectable hepatitis C virus (HCV) RNA in the liver cells and peripheral blood mononuclear cells, in the absence of serum HCV RNA, with or without presence of antibodies against HCV, defines occult hepatitis C [1–3]. Occult HCV infection can be presented with the following two clinical settings: (i) seronegative occult hepatitis C in patients without serological evidence of a prior exposure to HCV (HCV antibodies and serum HCV RNA negative) with persistently elevated liver enzymes, and (ii) seropositive occult hepatitis C in patients who had spontaneous or treatment induced clearance of HCV infection (HCV antibodies positive and serum HCV RNA negative). In both clinical presentations of occult hepatitis C, there is a persistence of HCV RNA in the liver cells and peripheral blood mononuclear cells [4–7].

The diagnosis and monitoring of HCV infection is based on the serologic detection of antibodies to HCV, followed by the detection of HCV RNA in serum with commercial diagnostic assays [8]. In the occult HCV infec-

tion, HCV RNA can be detected in the liver cells and peripheral blood mononuclear cells (PBMCs) but not in the serum. The reason for the absence of viral RNA in the serum of patients, whose virus replicated in their liver cells and PBMCs, could be explained by the persistence of very low levels of viremia not detectable with commercial diagnostic assays [9–11]. The PBMCs represent an extra hepatic site for HCV replication [12–14]. Testing for the presence of HCV RNA in PBMCs proved to be a reliable method of identification of occult HCV infection, without the need of a liver biopsy because of its invasive nature [15–18].

The presence of occult HCV infection represents a potential risk for the spread of HCV through transfusions, nosocomial transmission of the virus in dialysis units [19, 20] and also a risk for recurrence of the HCV infection after liver or renal transplantation, in patients treated successfully with antiviral therapy prior to transplantation [21, 22].

The standard treatment of HCV infection consists of a combination therapy of pegylated interferon alpha (PEG-IFN- α) and ribavirin (RBV) [23]. Monotherapy with a reduced dose of PEG-IFN- α is a standard treatment of chronic hepatitis C in hemodialysis patients, because

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ribavirin induced hemolytic anemia in this patient population [24–26]. Several small studies have demonstrated that the use of low-dose RBV in combination with interferon based therapy was feasible for treating chronic hepatitis C in hemodialysis patients. However, the use of RBV in hemodialysis patients has not been well studied and it should be used with extreme caution and close follow-up. [18, 26, 27]

OBJECTIVE

The aim of the study was to detect the persistence of occult hepatitis C in patients with treatment-induced clearance of the virus, using diagnostic assays with very low detection limit of viremia.

METHODS

Patients

A group of 13 hemodialysis patients and a group of 43 patients without a renal disease with treatment-induced clearance of hepatitis C virus infection were investigated in the study. The study was approved by the local Ethics Committee and a written informed consent was obtained from each study participant. The 13 patients on maintenance hemodialysis were treated only with pegylated interferon α -2a. It was administered subcutaneously, once a week at a standard dose of 135 μ g. The group of 43 patients without renal disease (non-renal) was treated with a combination therapy of pegylated interferon α -2a or α -2b and ribavirin. The PEG-IFN- α was administered subcutaneously once a week at a standard dose of 180 μ g for PEG-IFN- α -2a and 1.5 μ g/kg for PEG-IFN- α -2b. The ribavirin was administered daily at a dose of 1,000 mg for patients with body weight of less than 75 kg, and 1,200 mg for patients with body weight over 75 kg when combined with PEG-IFN- α -2a. When combined with PEG-IFN- α -2b, the ribavirin dose was 800 mg for patients with body weight of less than 65 kg, 1,000 mg for patients with body weight 65–85 kg, and 1,200 mg for patients with body weight greater than 85 kg. The treatment duration was 48 weeks for both groups of patients infected with HCV genotype 1, and 24 weeks for the patients infected with HCV genotype 2. All the patients had completed the antiviral treatment at least six months prior to enrollment in the study and they all achieved sustained viral response. The sustained viral response (SVR), defined as an absence of serum HCV RNA, six months after completion of the antiviral treatment [28], was confirmed by a reverse transcription polymerase chain reaction (RT-PCR) assay with a detection limit of 20 IU/ml. Peripheral blood samples in EDTA as an anticoagulant were obtained from each patient enrolled in the study.

HCV quantification

Hepatitis C virus RNA was extracted from plasma using QIAamp Viral RNA kit (Qiagen, Hilden, Germany) ac-

ording to the manufacturer's instructions. RT-PCR assay for HCV quantification was done with conventional HCV Real-TM Quant (Sacace Biotechnologies, Como, Italy) on Stratagene MX3005P real-time PCR system (Agilent Technologies, Edinburgh, UK) according to the manufacturer's instructions. Detection limit of the assay was 20 IU/ml.

Ultrasensitive detection of HCV RNA in plasma

All plasma samples which tested HCV RNA negative using the conventional real-time HCV Real-TM Quant test were re-extracted with QIAamp UltraSens Virus kit (Qiagen), following the manufacturer's instructions. The RNA extracted from 1 ml of plasma was afterwards precipitated with ethanol, suspended in RNase-free water, and placed into a single reaction to obtain maximum sensitivity. HCV RNA was detected by nested amplification of the 5' untranslated region (UTR), the region of the HCV genome [29]. The following primer pairs were used: 5'-GCAGAAAGCGTCTAGCCATGGCGT-3' (sense, KY-80) and 5'-CTCGCAAGCACCCCTATCAGGCAGT-3' (antisense, KY-78) for the RT-PCR round; and 6FAM-5'-CGGGAGAGCCATAGTGG-3' (sense, R-130fam) and 5'-CGGGAGAGCCATAGTGG-3' (antisense, R-290) for the nested round. The RT-PCR step was performed using Superscript III one-step PCR kit (Invitrogen) under the following conditions: reverse transcription at 58°C for 30 minutes, initial denaturation at 94°C for two minutes, and 55 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 68°C for one minute. For the nested round, 1 μ l of the RT-PCR product was used in a total volume of 25 μ l including 2 mM Mg²⁺, 200 μ M of each dNTP, 2 μ M of both primers, and 1.25 U Taq Gold polymerase (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The following conditions were used: initial denaturation at 95°C for 10 min, 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, and a final extension of 10 minutes at 72°C. Both positive and negative controls were included in each round. A total of 1 μ l of the nested PCR product was diluted in formamide (total volume 10 μ l), denaturated at 95°C for three minutes and separated with a capillary electrophoresis on ABI 3500 Genetic Analyzer (PE Applied Biosystems) using Gene Scan 500 LIZ Size Standard (Applied Biosystems). The sensitivity of the nested one-step RT-PCR amplification was determined by testing stepwise dilutions of the quantified HCV RNA plasma (4th World Health Organization international standard for HCV RNA, National Institute for Biological Standards and Control, code 06/102). The low HCV RNA dilutions (10, 5, 2, and 1 IU/ml) were tested in triplicates. The detection limit of the assay was 2 IU/ml.

Isolation of PBMCs

PBMCs were isolated with a density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and homogenized in Tri Reagent Solution (Ambion, Thermo Fisher Scientific).

Ultrasensitive detection of HCV RNA in PBMCs

HCV RNA was extracted from about 5×10^6 cells using the RNeasy Mini kit (Qiagen), following the manufacturer's instructions. The total RNA extracted from the PBMCs was precipitated with ethanol, suspended in RNase-free water, and used in a single reaction to maximize the sensitivity. HCV RNA was detected with a nested amplification of the 5' UTR region of the HCV genome by RT-PCR using the same materials and protocols as for the ultrasensitive detection in plasma. The detection limit of the assay was 2 IU/ml.

RESULTS

HCV genotype 1 was the cause of infection in 12 hemodialysis patients, while one patient was infected with HCV genotype 2 (Table 1). The demographic data of the hemodialysis patients and the distribution of HCV genotypes are presented in Table 1.

Sustained viral response after the antiviral treatment of HCV infection in hemodialysis patients was confirmed by a conventional RT-PCR assay with low detection limit of viremia (sensitivity: 20 IU/ml). HCV RNA was not detected in the PBMCs and plasma samples of hemodialysis patients either, by the use of the ultrasensitive RT-PCR assay (sensitivity: 2 IU/mL).

The group of 43 non-renal patients with SVR was also studied. HCV genotype 1 was the cause of infection in 95.3% of the patients, while dual infection with HCV genotype 1 and 3 was found in two patients (Table 2). The demographic data of the non renal patients and the distribution of HCV genotypes are presented in Table 2.

Achievement of sustained viral response in the non-renal patients was confirmed by the conventional RT-PCR assay with low detection limit of viremia (sensitivity: 20

IU/ml). When tested with the ultrasensitive RT-PCR assay (sensitivity: 2 IU/ml), HCV-RNA in the PBMCs and the plasma samples, was also not detected in any of the non-renal patients.

The hemodialysis patients and the patients without renal disease experienced treatment-induced clearance of the HCV infection confirmed by the ultrasensitive RT-PCR assay, which showed no detection of HCV RNA in the PBMCs and in the plasma samples. Thus, there was no detection of occult hepatitis C in the hemodialysis patients or in the patients without renal disease with achieved sustained viral response.

DISCUSSION

In the current clinical practice, patients with achieved sustained viral response following antiviral treatment, confirmed by commercial assays, are considered to have cleared the infection with HCV. However, data accumulated in the last decade demonstrate that the only effective approach to the diagnosis of HCV infection and its long-term persistence is the implementation of more sensitive HCV RNA diagnostic assays, as well as examination of the presence of the virus in the cells of the immune system. Searching for the presence of HCV viremia with more sensitive and ultrasensitive diagnostic assays, several investigator groups found that HCV RNA persisted at very low levels in the serum and PBMCs of patients with apparently spontaneous or treatment-induced resolution of the HCV infection [7, 30]. The study of Pham et al. [7] included 16 patients (five with spontaneous and 11 with treatment-induced resolution of HCV infection) who had undetectable serum HCV RNA tested with a commercial RT-PCR assay (sensitivity: 500 IU/ml). Low levels of viremia were detected in the serum and PBMCs of all patients using a highly sensitive RT-PCR-nucleic acid hybridization assay (sensitivity: 2 IU/ml). Radkowski et al. [30] investigated 11 HCV antibody positive patients who tested negative for serum HCV RNA with the commercial assay (sensitivity: 50 IU/mL). In the same study, low levels of HCV RNA were detected in the serum samples of six (55%) patients and in PBMCs of 11 (100%) patients, using a high-sensitivity RT-PCR assay (sensitivity: 2 IU/mL).

Our results did not reveal the presence of occult hepatitis C or persistence of HCV RNA at very low levels in the plasma or PBMCs of HD patients who achieved sustained viral response following antiviral treatment. Similar to our results, two studies which included hemodialysis patients who underwent renal transplantation after a successful treatment of HCV infection showed no presence of residual HCV RNA in their plasma samples and PBMCs. The first study included 16 patients who had achieved SVR after treatment with interferon- α during the HD [31]. At kidney transplantation, 38 months (range: 2–57 months) after interferon- α therapy, HCV viremia was negative in all patients. At the last follow-up after kidney transplantation, i.e. 22.5 months (range: 2–88 months), HCV viremia remained negative in all patients despite the immunosuppressive treatment. There

Table 1. Demographic data and HCV genotypes of the hemodialysis patients

Variable	Hemodialysis patients
Number, No. (%)	13
Sex, No. (%)	
Men	13 (100%)
Women	/
Age, year, mean \pm SD	47.7 \pm 10.2
HCV genotype, No. (%)	
G1	12 (92.3%)
G2	1 (7.7%)

Table 2. Demographic data and HCV genotypes of the non-renal patients

Variable	Patients without a renal disease
Number, No. (%)	43
Sex, No. (%)	
Men	30 (69.8%)
Women	13 (30.2%)
Age, year, mean \pm SD	40.0 \pm 10.6
HCV genotype, No. (%)	
G1	41 (95.3%)
G1 + G3	2 (4.7%)

was no detection of HCV RNA in the examined PBMCs of eight transplanted patients. The second study assessed the persistence of HCV infection in 26 renal transplant patients on immunosuppressive treatment, who had spontaneous (No. = 10) or treatment-induced clearance (No. = 16) of the HCV infection while on HD [29]. No biochemical or viral relapse was detected in the studied patients during the median post-transplant follow-up of 10.5 years (range: 2–16 years). HCV RNA was not detected in any of the patient's plasma samples, repeatedly taken during the follow-up period (average: 5 times; range: 1–15 times), using a conventional RT-PCR assay (sensitivity: 15 IU/mL). Residual HCV RNA was also not detected in their plasma samples and PBMCs (unstimulated and stimulated) with an ultra-sensitive RT-PCR assay (sensitivity: 2 IU/mL).

Our study results also confirmed effective clearance of HCV from the plasma and PBMCs of patients without renal disease who achieved SVR following standard antiviral treatment. Occult hepatitis C was also not detected in other studies involving patients without renal disease with achieved SVR after antiviral treatment. George et al. [32] investigated 150 patients with SVR after HCV infection treatment and followed them up for five years, for evidence of viral relapse. The presence or absence of HCV RNA in their sera was determined by RT-PCR methodology with a sensitivity of 29 IU/mL. There was no detection of HCV RNA in the patients' serum samples, confirming that there was no evidence of HCV RNA persistence. Morisco et al. [33] evaluated the long-term eradication of HCV infection in 150 treated patients with achieved SVR. The median follow-up was 8.6 years (range 2–19.9 years). Presence of HCV RNA in the serum was not detected in all study participants, using the RT-PCR assay with sensitivity of less than 50 IU/mL, when testing at least four blood samples taken at different time points.

The concept of occult hepatitis C is still a matter of debate. Some studies detected occult hepatitis C and other studies did not detect it in patients with treatment-induced clearance of HCV infection. The disagreement between these studies could be associated with the differences of the criteria used to define sustained viral response after

antiviral treatment. There were differences in the detection limit of the RT-PCR assays used to determine the sustained viral response, ranging from 15 IU/ml to 500 IU/ml. Therefore, HCV RNA would have probably been detected in the serum of patients with occult hepatitis C, if RT-PCR assays with a low detection limit of viremia had been used to check achievement of sustained viral response.

CONCLUSION

Occult hepatitis C was described as a phenomenon characterized by undetectable HCV RNA in the serum tested with commercial assays, but with detection of viral RNA in PBMCs and/or liver cells. The "absence" of HCV RNA in the serum could be explained with the persistence of very low levels of viremia, frequently escaping detection by commercial assays. When HCV RNA was undetectable in the serum using assay with high sensitivity, there was also absence of HCV RNA in the peripheral blood mononuclear cells in patients with treatment-induced viral clearance.

The implementation of assays with high sensitivity, capable of detecting low levels of viral RNA, for clinical and population-based examination, should be the only effective approach to determine a long-term sustained viral response in patients treated with antiviral therapy.

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Претрага за присуство окултног хепатитиса Ц код пацијената са терапијском елиминацијом вируса применом ултрасензитивног теста

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КРАТАК САДРЖАЈ

Увод Окултни хепатитис Ц је дефинисан присуством вируса хепатитиса Ц у мононуклеарним ћелијама периферне крви и/или хепатоцитима, при негативним налазом вирусне рибонуклеинске киселине (РНК) у серуму.

Циљ рада Циљ овог рада је утврдити присуство окултног хепатитиса Ц код пацијента на хроничној хемодијализи и код пацијента без бубрежне болести, код којих је постигнута терапијска елиминација вируса, помоћу примене ултрасензитивног теста за детектовање вирусне РНК.

Методe рада У студији је било укључено 13 пацијента на хемодијализи и 43 пацијента без бубрежне болести, код којих је постигнута елиминација вируса хепатитиса Ц помоћу антивирусног третмана. Пацијенти на хемодијализи били су лечени само пегилираним интерфероном алфа, док су пацијенти без бубрежне болести били лечени комбинацијом пегилираног интерферона алфа и рибавирина.

Потенцијално присуство вирусне РНК у мононуклеарним ћелијама периферне крви и у плазми пацијента је испитивано помоћу ултрасензитивног теста за детектовање вирусне РНК (сензитивност од 2 IU/ml).

Резултати Применом ултрасензитивног теста, вирусна РНК није била откривена ни у мононуклеарним ћелијама периферне крви нити у плазми хемодијализних пацијента и пацијента без бубрежне болести.

Закључак Применом ултрасензитивног теста за детектовање вирусне РНК, који се користио за утврђивања успешности терапије код хепатитиса Ц, окултни хепатитис Ц није био откривен ни код хемодијализних, нити код пацијената без бубрежне болести.

Кључне речи: вирус хепатитиса Ц; лечење; пегилиран интерферон алфа, рибавирин; хемодијализа; мононуклеарни леукоцити

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