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High prevalence of occult hepatitis C virus infection in patients with primary and secondary glomerular nephropathies

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The association of hepatitis C virus (HCV) infection and glomerulonephritis is well known. However, the relationship between immune-mediated glomerulonephritis and occult HCV, characterized by the presence of HCV-RNA in liver or in peripheral blood mononuclear cells in the absence of serological markers, is unknown. We tested this in 113 anti-HCV-negative patients; 87 with immune-mediated glomerulonephritis and 26 controls with hereditary glomerular nephropathies. All patients were serum HCV-RNA negative by conventional real-time PCR. Significantly, occult HCV-RNA (detectable viral RNA in peripheral blood mononuclear cells or in serum after ultracentrifugation) was found in 34 of 87 patients with immune-mediated glomerulonephritis versus 1 of 26 control patients. The serum creatinine levels were significantly higher in patients with immune-mediated glomerulonephritis with than in those without occult HCV (1.5 versus 1.1 mg/dl, respectively). A multivariate analysis adjusted for gender showed a significantly increased risk of occult HCV in patients with immune-mediated glomerulonephritis versus the controls (odds ratio of 13.29). Progression to end-stage renal disease tended to be faster in patients with immune-mediated glomerulonephritis and occult HCV than in the negative cases. Thus, occult HCV is strongly associated with immune-mediated glomerulonephritis and may have a role in the progression of the disease.

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Hepatitis C virus (HCV) is a single-stranded RNA virus that replicates by the synthesis of a complementary RNA molecule (antisense HCV-RNA strand). Although liver is the main target for HCV infection, a significant proportion of the HCV-associated morbidity is due to extrahepatic pathologies.[1] These extrahepatic manifestations may occur due to a generalized autoimmunity induced by the virus or a direct action of HCV, as HCV-RNA has been detected in several extrahepatic cell types and organs including the kidney.[2,3]

Different epidemiological studies have shown that HCV infection is closely associated with several chronic kidney diseases, such as type I membranoproliferative glomerulonephritis with and without cryoglobulinemia, type III membranoproliferative glomerulonephritis, and membranous nephropathy.[4–6] The common feature of these HCV-associated renal diseases is the existence of HCV immune complexes in renal tissue.[7–9]

The diagnosis of HCV infection is based on the detection of antibodies against HCV proteins (anti-HCV) and HCV-RNA in serum.[10] However, the use of highly sensitive nucleic acid amplification assays has demonstrated the presence of HCV-RNA in serum of some anti-HCV-negative patients. This status, termed 'seronegative' HCV infection, was found not only in certain clinical conditions such as HIV infection and hemodialysis but also in cryptogenic hepatitis and in blood and organ donors.[11–20] Another form of seronegative HCV infection named occult HCV was identified in patients with chronic liver disease of unknown origin.[21] This occult HCV infection is characterized by the detection of HCV-RNA in liver in the absence of anti-HCV and HCV-RNA in serum even on using highly sensitive techniques. Furthermore, a proportion of patients with occult HCV may have HCV-RNA in peripheral blood mononuclear cells (PBMCs) and very low levels of serum HCV-RNA may be detected after ultracentrifugation of large volumes of serum to concentrate viral particles.[22] These approaches can be useful for the diagnosis of occult HCV when a liver biopsy is not available. Using

these techniques, occult HCV infection was also described in cryptogenic liver cirrhosis, hepatocellular carcinoma, lymphoproliferative disorders, hemodialysis patients with abnormal values of liver enzymes of unknown origin, and even in healthy subjects without evidence of hepatic disease.[23–29]

On the basis of the above premises, this study aimed to determine the prevalence and the possible clinical significance of occult HCV infection in a population of anti-HCV-negative patients with immune-mediated glomerulonephritis (IMGN).

RESULTS

This prospective study included a total of 113 adult patients with glomerulonephritis who were divided into two groups. One of the groups comprised 87 cases with IMGN (65 patients with primary and 22 with secondary glomerulonephritis) and the other group (control group) had 26 patients with hereditary glomerular nephropathies (HGN) (Table 1) When comparing the demographic and clinical features of the two groups of patients included in the study, it was found that the proportion of women (20/26: 77%) and the duration of the renal disease (233 ± 99.5 months) were significantly higher in the group of patients with HGN than in patients with IMGN (45/87: 52%, $P = 0.025$ and 103.8 ± 91.5 months, $P = 0.001$, respectively). By contrast, the number of patients who reported risk factors for acquisition of HCV infection (blood transfusions, tattoos or piercings, or household contact with a chronic HCV carrier) was significantly higher ($P = 0.007$) in the IMGN group (25/87: 29%) than in the control group (1/26: 4%). No other differences were found between both groups of patients (Table 2).

Table 1 | Demographic and clinical characteristics of the 113 patients

Age (year; mean ± s.d.)	49.3 ± 17.8
Female (*n* (%))	65 (57.5)
Duration of renal disease (months; mean ± s.d.)	133.5 ± 107.8
Serum creatinine (mg/dl; mean ± s.d.)	1.36 ± 1.27
Creatinine clearance (ml/min per 1.73 m^2; mean ± s.d.)	88.6 ± 42.9
Cockroft–Gault (ml/min; mean ± s.d.)	83.6 ± 40.1
Primary glomerular nephropathy (n *(%))*	
Idiopathic nephrotic syndrome[a]	26 (23)
Membranous nephropathy	20 (17.7)
Immunoglobulin A nephropathy	14 (12.4)
Membranoproliferative glomerulonephritis[b]	3 (2.6)
Immunotactoid nephropathy	2 (1.8)
Secondary glomerular nephropathy (n *(%))*	
Lupus nephropathy	15 (13.3)
Anti-neutrophil cytoplasmic antibody-positive vasculitis	7 (6.2)
Hereditary glomerular nephropathy (n *(%))*	
Alport syndrome	13 (11.5)
Thin basement membrane disease	8 (7.1)
Benign familial hematuria	5 (4.4)

[a]Includes minimal change nephrotic syndrome, focal and segmental glomerulosclerosis, and IgM nephropathy.
[b]Types I, II, and III; normal values for serum creatinine ⩽1.3 mg/dl; normal values for Cockroft–Gault >90 ml/min.

Serum HCV-RNA was tested by conventional real-time PCR (with 250 µl of serum) in the 113 patients and all of them were negative. When detecting sense HCV-RNA strand in PBMC samples, it was found that 29/87 (33%) patients with IMGN who were serum HCV-RNA negative by conventional PCR technique had HCV-RNA in PBMCs with a mean load ± s.e.m. of $4.7\times10^4 \pm 1.7\times10^4$ copies/µg of total RNA. In 17 of these 29 patients (59%) the antisense HCV-RNA strand (HCV replication) was also detected in PBMCs (mean ± s.e.m.: $2.6\times10^3 \pm 1.8\times10^3$ copies/µg of total RNA). In addition, HCV-RNA was detected in serum after ultracentrifugation in another five additional patients (mean ± s.e.m.: 99 ± 16 copies/ml). No patient was simultaneously positive for HCV-RNA in PBMCs and in serum after ultracentrifugation. Thus, an occult HCV infection (HCV-RNA in PBMCs or in serum after ultracentrifugation) was diagnosed in 34/87 (39%) anti-HCV-negative patients with IMGN. By contrast, occult HCV infection was identified only in PBMCs of 1/26 (3.8%) patients with HGN ($P = 0.001$). All the negative controls included in the PCR assays were always negative and the results of HCV-RNA detection performed in a blind fashion by different operators on different days were identical in all cases.

The sequence analysis of the HCV-core region isolated from PBMCs of seven randomly selected patients and from serum (after ultracentrifugation) of two other patients showed that HCV isolates belonged to genotype 1b. The phylogenetic tree proved that, in each patient, HCV clones clustered together into the same branch but separately from

Table 2 | Demographic and clinical features of the two groups of patients

	Immune-mediated glomerulonephritis ($n = 87$)	Hereditary glomerular nephropathy ($n = 26$)	*P*
Age (year; mean ± s.d.)	49.8 ± 18.6	47.6 ± 14.5	0.577
Female (*n* (%))	45 (52)	20 (77)	0.025
Duration of renal disease (mo.; mean ± s.d.)	103.8 ± 91.5	232.8 ± 99.5	0.001
Serum creatinine (mg/dl; mean ± s.d.)	1.24 ± 0.7	1.78 ± 2.3	0.055
Creatinine clearance (ml/min per 1.73 m^2; mean ± s.d.)	87.5 ± 41.7	92.5 ± 47.7	0.611
Cockroft-Gault (ml/min; mean ± s.d.)	82.7 ± 38	86.4 ± 47.2	0.694
Risk factors for acquisition of hepatitis C virus (*n* (%))	25 (29)	1 (4)	0.007

Normal values for serum creatinine ⩽1.3 mg/dl; normal values for Cockroft–Gault >90 ml/min. Risk factors for acquisition of hepatitis C virus: blood transfusions, tattoos/piercings, household contact with a chronic hepatitis C virus carrier.

the HCV clones of the other patients, indicating that no cross-contamination among samples occurred (Figure 1).

By univariate analysis, it was found that the incidence of occult HCV infection was significantly higher in patients with idiopathic nephrotic syndrome, membranous nephropathy, Immunoglobulin A nephropathy, lupus nephropathy, and anti-neutrophil cytoplasmic antibody-positive vasculitis in comparison with patients with HGN (see Table 3).

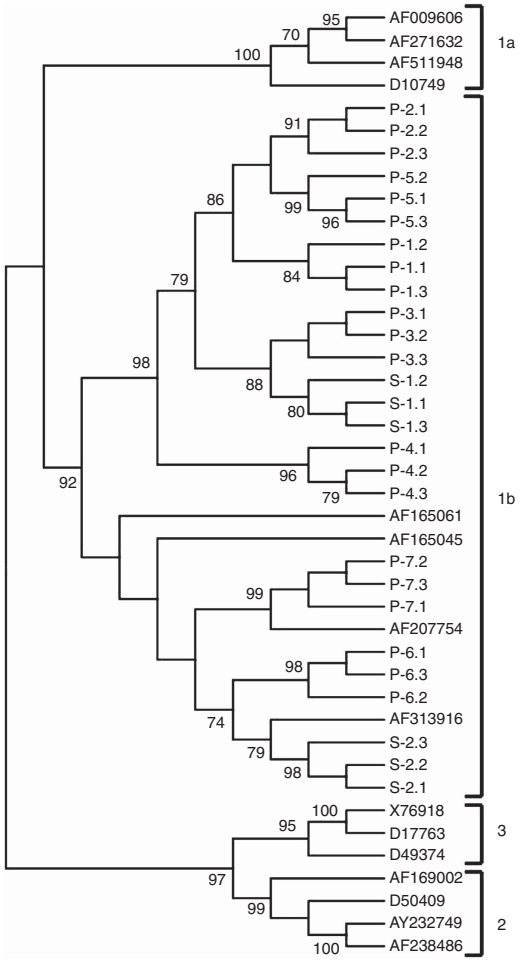

Figure 1 | Phylogenetic tree constructed with the hepatitis C virus (HCV)-core nucleotide sequences isolated from peripheral blood mononuclear cells (PBMCs) of seven randomly selected patients, designated by 'P', and from the serum, after ultracentrifugation, of two different patients, designated by 'S' (GenBank accession numbers KF889406-KF889432), and those corresponding to HCV genotypes 1, 2, and 3. Bootstrap values ⩾70 obtained after 1000 replicates of the data sheet are shown in the nodes of the tree.

In the group of IMGN patients, age, duration of the renal disease, levels of liver enzymes, and risk factors for acquiring HCV infection were comparable among patients with and without occult HCV infection (Table 4). However, the frequency of female gender was significantly lower ($P = 0.014$) in the group of patients with occult HCV (12/34: 35%) compared with that in the negative group (33/53: 62%). In addition, patients with occult HCV infection had significantly higher levels of serum creatinine compared with those without occult HCV (1.5 ± 0.9 vs. 1.1 ± 0.4 mg/dl, $P = 0.015$). Recorded data on the presence of rheumatoid factor were only available for 47 patients (19 of them with occult HCV), being positive in 1/19 (5%) cases with occult HCV infection and in 3/28 (11%) of those without occult HCV ($P = 0.638$). Cryoglobulins had been only tested in 11 patients (six with and five without occult HCV infection) and all of them were negative.

The multivariate analysis adjusted for gender revealed an increased risk of having an occult HCV infection in the group of patients with IMGN (odds ratio = 13.29; 95% confidence interval: 1.695–104.229; $P = 0.014$).

After 42.5 ± 8.5 months of the study entry, 81 patients with IMGN (30 with occult HCV infection) were still attending our Nephrology Departments. The remaining six patients (four with occult HCV) were lost for follow-up. Final serum creatinine levels did not differ significantly among patients with and without occult HCV infection (2.0 ± 1.9 vs. 1.4 ± 2.1 mg/dl, respectively), although creatinine clearance was significantly lower ($P = 0.032$) among patients with occult HCV (68.2 ± 45.1 ml/min per 1.73 m^2) than in those without occult infection (89.7 ± 39.6 ml/min per 1.73 m^2). However, the rate of renal function decline was comparable in both groups (data not shown). Relative to clinical outcome, the frequency of end-stage renal disease progression tended to be higher in patients with occult HCV (5/30:17%) compared with that in the negative ones (2/51: 4%) but with no statistically significant difference ($P = 0.062$). A total of 24 patients (seven with occult HCV infection) gave their consent to be retested for HCV-RNA in

Table 3 | Prevalence of occult HCV infection according to the type of glomerulonephritis

	Number of patients	Occult HCV positive (%)	Odds ratio	95% Confidence interval	*P*
Idiopathic nephrotic syndrome[a]	26	8 (31)	11.11	1.28–96.86	0.029
Membranous nephropathy	20	8 (40)	16.67	1.97–148.90	0.012
Immunoglobulin A nephropathy	14	7 (50)	25.0	2.62–238.79	0.005
Membranoproliferative glomerulonephritis[b]	3	1 (33)	12.50	0.55–284.12	0.113
Immunotactoid nephropathy	2	0 (0)	0.00	0.00	0.999
Lupus nephropathy	15	7 (47)	21.88	2.33–205.78	0.007
Anti-neutrophil cytoplasmic antibody-positive vasculitis	7	3 (43)	18.75	1.54–227.78	0.021

[a]Includes minimal change nephrotic syndrome, focal and segmental glomerulosclerosis, and IgM nephropathy.
[b]Types I, II, and III.

Table 4 | Comparison of patients with and without occult hepatitis C virus infection in immune-mediated glomerulonephritis

	Immune-mediated glomerulonephritis			
	Positive occult hepatitis C virus ($n = 34$)	Negative occult hepatitis C virus ($n = 53$)	P^a	Hereditary glomerular nephropathy ($n = 26$)
Age (year; mean ± s.d.)	51.6 ± 21.4	48.7 ± 16.7	0.502	47.6 ± 14.5
Female (*n* (%))	12 (35)	33 (62)	0.014	20 (77)
Duration of renal disease (months; mean ± s.d.)	100.2 ± 103.9	106.1 ± 83.6	0.774	232.8 ± 99.5
Serum creatinine (mg/dl; mean ± s.d.)	1.5 ± 0.9	1.1 ± 0.4	0.015	1.78 ± 2.3
Creatinine clearance (ml/min per 1.73 m^2; mean ± s.d.)	78.6 ± 38	93.2 ± 42.8	0.113	92.5 ± 47.7
Cockroft-Gault (ml/min; mean ± s.d.)	73.7 ± 38	88.5 ± 37.2	0.096	86.4 ± 47.2
Risk factors for acquisition of HCV (*n* (%))	12 (35)	13 (25)	0.279	1 (4)
Aspartate aminotransferase (IU/l, mean ± s.d.)	22.1 ± 6.2	21.0 ± 5.2	0.377	21.0 ± 11.5
Alanine aminotransferase (IU/l, mean ± s.d.)	20.4 ± 11	20 ± 9	0.825	20 ± 13.2
Gamma-glutamyl transpeptidase (IU/l, mean ± s.d.)	25.4 ± 12.8	34.6 ± 34.1	0.078	28.4 ± 35

Risk factors for acquisition of hepatitis C virus: blood transfusions, tattoos/piercings, household contact with a chronic hepatitis C virus carrier.
Aspartate aminotransferase (normal value <40 IU/l); Alanine aminotransferase (normal value <40 IU/l); gamma-glutamyl transpeptidase (normal value <45 IU/l); normal values for serum creatinine ⩽1.3 mg/dl; normal values for Cockroft-Gault >90 ml/min.
[a]*P*-values corresponding to the comparison of immune-mediated glomerulonephritis patients with and without occult hepatitis C virus.

PBMCs and in serum after ultracentrifugation. This HCV-RNA detection was also done in a blinded fashion. The 17 patients without occult HCV remained HCV-RNA negative, both in PBMCs and in serum after ultracentrifugation. Of the seven patients with occult HCV, initially, one had viral RNA in serum (after ultracentrifugation) and the other six patients were HCV-RNA positive in PBMCs. In the follow-up sample, all the seven patients were HCV-RNA negative in serum after ultracentrifugation. However, viral RNA was detected in the PBMCs of the seven patients, confirming the presence of an occult HCV infection.

DISCUSSION

Previous reports have shown an association between HCV infection and glomerulonephritis.[4–6] The routine laboratory diagnosis of this HCV-related glomerulonephritis is based on the presence of conventional HCV-serological markers (anti-HCV and HCV-RNA). However, in the past years, the existence of occult HCV infection (presence of viral RNA in liver and in PBMCs with undetectable anti-HCV and serum HCV-RNA) has been documented in different populations, including patients with cryptogenic hepatitis, hemodialysis patients, and healthy subjects without evidence of liver disease.[21,23–29]

This work was aimed to investigate the prevalence of occult HCV infection in anti-HCV and serum HCV-RNA-negative patients with IMGN. We have found that 39% of these patients had an occult HCV infection, as demonstrated by the detection of HCV-RNA in PBMCs or in serum after ultracentrifugation. The PCR results were considered reliable because (i) the large number of negative controls included in each PCR run were always negative, (ii) laboratory personnel were blinded to the clinical status of patients and identical results were obtained by different operators when testing the samples on different days, and (iii) the results of the phylogenetic analysis performed after the amplification of the HCV-core region, which was a different region than that amplified for the assessment of HCV-RNA (the 5' noncoding region) in all the samples included in the study. Finally, the presence of occult HCV infection was confirmed when repeating HCV-RNA detection in samples collected 42.5 ± 8.5 months after the initial ones.

The prevalence of occult HCV infection in patients with IMGN (39%) was significantly higher ($P = 0.001$) than that found in the group of patients with HGN (3.8%). Furthermore, the multivariate analysis showed that the risk of having an occult HCV infection was 13 times higher for patients with IMGN as compared with HGN, so all these data suggest that occult HCV infection is clearly associated with glomerular diseases.

In patients with classical HCV-associated glomerular nephropathies, the pathogenesis of the kidney lesion is related to the deposition of HCV immune complexes in the glomeruli.[7–9] These immune complexes can be taken up by mesangial cells, where viral RNA can trigger the production of interferons and proinflammatory cytokines via Toll-like receptor 3–dependent and –independent mechanisms.[30,31] Patients with occult HCV infection have neither detectable anti-HCV nor detectable serum HCV-RNA by conventional methods and therefore it could be argued that occult HCV infection does not have a role in the kidney injury of our studied patients. However, it has been reported that in an anti-HCV-positive patient with glomerulonephritis but negative to serum HCV-RNA for years, renal injury was likely to be virus-induced because of the presence of HCV-NS3 antigen in the kidney tissue of the patient.[32] Moreover, a recent work describes detection of HCV antigens and visualization of HCV particles in the renal tissue of 1.8% of anti-HCV-negative patients with glomerulonephritis, although the status of HCV-RNA in serum or PBMCs is not provided.[33] This percentage of occult HCV is lower than that found in our study, but it should be considered that immunohistochemistry is a lesser sensitive technique than HCV-RNA detection. Therefore, taking together all these previouly reported data and our results, it can be assumed that occult HCV infection may be the underlying cause of a

proportion of primary and secondary glomerular nephropathies with negative serological viral markers. Although in the present work immunohistochemistry or virologic detection in the kidney biopsies (as no properly stored material was available) were not performed, our data suggest a possible direct pathogenic role of occult HCV infection in the renal damage. In support of this hypothesis is the fact that IMGN patients with occult HCV infection had significantly worse renal function (serum creatinine levels and creatinine clearance values) than the IMGN patients without occult HCV. Although the renal function declining rate was similar between both groups, the frequency of progression to end-stage renal disease tended to be higher in patients with occult HCV than in the negative cases, indicating that occult HCV may be a negative prognostic factor, as occurs with the classical hepatitis C infection in patients with IMGM.[34] Finally, occult HCV infection could explain why interferon therapy is of benefit, inducing clinical and laboratory responses, in some HCV-negative patients with essential mixed cryoglobulinemia.[35,36] Therefore, detection of occult HCV infection provides the rationale basis for administration of antiviral therapy in anti-HCV-negative glomerular nephropathies.

In summary, occult HCV infection may be involved in the pathogenesis of a significant proportion of IMGN but further studies and a long-term follow-up of these patients must be performed to confirm the clinical and pathological implications of this finding.

MATERIALS AND METHODS

From June 2009 to January 2012, 87 patients with primary and secondary glomerular nephropathies and 26 patients with hereditary glomerular nephropathy were enrolled in the study. The diagnosis of the renal disease was based on a kidney biopsy, except for the cases of benign familial hematuria diagnosed by clinical and familial data. All patients had to be anti-HCV-negative, as well as hepatitis B surface antigen and anti-HIV negative by routine commercial tests (VITROS Anti-HCV Assay, Ortho Clinical Diagnostics, Raritan, NJ; Enzygnost hepatitis B surface antigen 5.0 and Enzygnost Anti-HIV 1/2 Plus Siemens Healthcare Diagnostics, Marburg, Germany). The study was approved by the Ethic Committee of the Hospital Universitario La Paz and was conducted according to the Declaration of Helsinki. A written informed consent was obtained from each patient.

Laboratory procedures

Blood samples were collected from all patients at the time of entry into the study. Upon arrival of the blood to the laboratory, serum samples were obtained from clotted blood, made into aliquots, and stored at $-80\,^{\circ}C$, whereas PBMCs were isolated from anti-coagulated blood by Biocoll (Biochrom, Berlin, Germany) density gradient centrifugation and stored at $-20\,^{\circ}C$ in RNAlater solution (Ambion, Austin, TX). HCV-RNA detection was performed over the inclusion period by laboratory personnel who were blinded to the clinical status of the patients. Each PCR run included a maximum number of six samples along with negative controls (repeatedly HCV-RNA-negative sera and PBMC samples from five healthy volunteers) and reagent blanks in which total RNA was replaced with PCR-grade water. All negatives controls were co-prepared with the samples and accompanied the samples through the entire PCR process. In addition, to avoid PCR contamination, the guidelines of Kwok and Higuchi[37] were strictly observed. Finally, all HCV-RNA-positive samples, as well as randomly selected negative samples were retested again on different days by another person who was blinded to previous PCR results.

HCV-RNA detection

Total RNA was extracted from 250 µl of serum using Trizol LS Reagent (Invitrogen, Carlsbad, CA), and after precipitation the pellet was dissolved in diethyl-pyrocarbonate water. Another 2 ml of the serum was ultracentrifuged over a 10% sucrose cushion at $100{,}000\times g$ for 17 h at $4\,^{\circ}C$. The pellet was dissolved in 250 µl of TE buffer (Tris-HCl 10 mmol/l, EDTA 10 mmol/l; pH 7.5); total RNA was isolated with Trizol LS Reagent (Invitrogen), precipitated, and the pellet dissolved in diethyl-pyrocarbonate-treated water. Total RNA from PBMCs was isolated with SV Total RNA Isolation System (Promega, Madison, WI). After precipitation, pellets were dissolved in diethyl-pyrocarbonate-treated water and RNA concentration was determined by spectrophotometry.

The detection of the 5' noncoding region of the sense HCV-RNA strand (using 5 µl of total RNA isolated from 250 µl of serum or from 2 ml of ultracentrifuged serum, or using 0.5 µg of total RNA from PBMCs) and of the antisense HCV-RNA strand (in PBMC), was performed by a strand-specific real-time RT-PCR using the thermostable enzyme Tth for the synthesis of the corresponding cDNA at high temperature as described.[22] Real-time PCR was performed with FRET probes in a LightCycler (Roche Diagnostics, Mannheim, Germany) with 2 µl of cDNA in a final volume of 20 µl, using the LightCycler FastStart DNA Master HybProbe Kit (Roche Diagnostics), as reported.[22] A standard curve constructed with 10-fold dilutions of a synthetic HCV-RNA of sense polarity was used for quantification of sense HCV-RNA strand in PBMCs and in serum. As reported before, the sensitivity of this assay was of three HCV-RNA copies per reaction.[22]

Sequence analysis

To further assure the specificity of the results, partial amplification of the HCV-core gene (302 nucleotides) was performed as described[21] in total RNA isolated from PBMCs of seven randomly selected patients with occult HCV infection and from the serum, after ultracentrifugation, of two other patients. PCR products were cloned into the pCR II TOPO vector (Invitrogen) and clones were automatically sequenced. Nucleotide sequences of HCV genotypes 1–3 were retrieved from GenBank and a phylogenetic tree was constructed by the Neighbor-Joining method using the MEGA software version 4.0.[38] The data set was bootstrap-resampled 1000 times to ascertain support for major branches of the tree.

Statistical analysis

Categorical variables were compared using chi-squared test or the Fisher's exact test, as appropriate. Continuous variables were compared using the Student's *t*-test or the Mann–Whitney's *U*-test. A multivariate logistic regression analysis was performed to determine independent associated factors of the absence or presence of occult HCV infection.

DISCLOSURE

All the authors declared no competing interests.

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