

Research Article

Recurrence of occult hepatitis B virus infection in a recipient of a liver transplant for HCV-related cirrhosis: full length genome, mutations analysis and literature review

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ABSTRACT

The outcome of liver transplant recipients in HCV chronic carriers with Anti-HBc only concerning occult HBV infection is unknown. We report here the case of a patient who underwent liver transplantation (LT) for cirrhosis post chronic hepatitis C who received an allograft from a donor with no marker of hepatitis B infection. After LT, HBV DNA was detected in the serum in the absence of HBsAg while HCV RNA remained negative. To determine the origin of this occult HBV infection, we retrospectively examined stored serum and liver tissue, pre and post-transplantation, for HBV DNA by PCR. A stored liver biopsy of the donor before transplantation was also tested. HBV DNA was detected in the pre-transplant liver but not in the donor liver. HBV viral load quantified by real time PCR after LT ranged from about 10^2 to 5×10^3 HBV DNA copies/mg of liver, while in sera, concentrations ranged from 10^2 to 3×10^3 HBV DNA copies/ml. All PCR products in the S gene from liver and sera were sequenced. Analysis of sequences showed the presence of an HBV strain genotype D. The nucleotide homology between the patient's HBV strains before and after LT was 96 % across the analyzed regions. Full length HBV genomes were amplified from the sera using Rolling Circle Amplification and then sequenced. Analysis of sequences confirmed the genotype D, but did not show obvious mutations that could contribute to HBsAg seronegativity and low HBV viral replication. Factors leading to occult HBV infection are still unclear, but it is well establish that occult HBV infection is frequent in HCV patients. This underlines the role of extra hepatic sites for HBV replication, potentially lymphocytes acting as "reservoirs".

Keywords: Occult HBV infection, Chronic hepatitis C, HBV-DNA, HBsAg, Liver transplantation

INTRODUCTION

Hepatitis C virus (HCV) and hepatitis B virus (HBV) are the most common causes of chronic liver disease worldwide. Co-infection with both viruses may commonly occur because of shared routes of infection. Patients with dual HBV and HCV have more severe liver

disease, and are at an increased risk for progression to hepatocellular carcinoma (HCC). End-stage liver disease secondary to chronic HCV and/ or HBV infection is the leading indication for liver transplantation (LT).

Active hepatitis B infection was in the past considered a relative contraindication for liver transplantation in

chronic liver failure due to the high rate of early recurrent disease under immunosuppression.¹ An effective antiviral treatment strategy for preventing of graft failure is essential for successful transplantation in this setting. Hepatitis C is a major indication for liver transplantation and accounts for approximately 50% of LT in the United States and Europe.²

Prophylactic antibody treatment is only given in serologically confirmed hepatitis B co-infected patients. However, there are now reports of serologically negative patients who have HBV DNA in serum and liver.^{3,4} Nowadays, occult HBV infection is recognized as a world-wide phenomenon, although its distribution may reflect the general prevalence of HBV in various geographic areas and in different populations.

Several studies have reported that occult HBV infections aggravate liver disease and fasten progression to cirrhosis and HCC.^{5,6} There is a fairly general agreement in considering that HCV infected patients is the clinical category with the highest prevalence of occult HBV.⁷⁻⁹ Moreover, occult HBV infection is frequently associated with the presence of anti-HBc⁵ and in patients under immunosuppression, recurrence or reactivation of occult HBV infection was reported in anti-HBc positive patient with undetectable HBsAg.^{10,11}

Considering that HBsAg is frequently used as the primary marker of ongoing HBV infection, the presence of chronic liver failure in hepatitis C patients with occult hepatitis B infection and their assessment for liver

transplantation may be underestimated and so far not detected.

Here, we describe a case of occult HBV infection in a liver grafted for HCV-related cirrhosis which persisted after liver transplantation while HCV infection was cleared through the removal of the cirrhotic infected liver.

METHODS

The patient and serological tests

The patient is a 60-year-old man who underwent Liver Transplantation (LT) for cirrhosis post chronic hepatitis C. At that time, he was HCV RNA positive and investigation of serologic parameters for HBV showed that he was negative for hepatitis B surface antigen (HBsAg) and anti-HBs (HBs antibody), but positive for anti-HBc IgG. The relevant biochemical and virological parameters for the period 2008-2010 are shown in Table 1. For the liver donor, routine screening for all hepatitis virus and HIV was negative. The donor had normal liver function tests. Before transplantation, sera and liver samples from the recipient and donor were collected and stored at -70°C. After transplantation, the patient was followed-up every month in the outpatient clinic for the first 6 months, 3-6 months thereafter until 4 years after liver transplantation. Liver function tests, HBV serology and HCV RNA were routinely tested.

Table 1: Biochemical and virological follow-up of the liver transplant recipient.

Data	Apr *2008	May 2008	Aug 2008	Nov 2008	Jan 2009	Mar 2009	Mar 2009	Jan 2010	Apr 2010	Aug 2010	Sep 2010
ASAT (UI/L)	NT	20	20	18	26	24	46	32	24	18	20
ALAT (UI/L)	NT	18	16	16	26	28	54	44	26	16	16
T Bil (mg/L)	NT	5	5,5	5	4	5	5	7	5	5	6
D Bil (mg/L)	NT	0	0	0	0	0	0	0	0	0	0
γGT (UI/L)	NT	16	NT	18	16	14	24	20	12	12	10
αFP (ng/mL)	NT	NT	3	NT	NT	2	NT	NT	4	NT	3
HBsAg	-	-	-	-	-	-	+	-	-	-	-
Anti-HBs	-	-	-	-	-	-	-	-	-	-	-
Anti-HBc	+	+	+	+	+	+	+	+	+	+	+
AgHBe	NT	-	NT	-	-	NT	-	-	NT	NT	NT
Anti-HBe	NT	-	NT	-	-	NT	-	-	NT	NT	NT
Anti-HCV	+	-	-	-	-	-	-	-	-	-	-
HCV-RNA	NT	TND									

ASAT, aspartate aminotransferase n.v. 0-40 UI/L; ALAT, alanine aminotransferase n.v. 0-40; T Bil: total bilirubin n.v. up to 12 mg/L; D Bil: direct (conjugated) bilirubin n.v. up to 3 mg/L; γGT: γ-glutamyl transferase n.v. 0-35 UI/L; αFP, α-fetoprotein n.v. 15 ng/mL. NT: not tested; TND: target not detected.

HBsAg detection was carried out with MonoLisa HBsAg Plus (Bio-Rad). Anti-HBs antibodies (Anti-HBsAb) were tested by ECLIA (Roche). Total anti-HBc (hepatitis B core antibody or anti-HBc) was evaluated by ECLIA (Roche). HCV antibodies were tested using ELISA (Bio-Rad). HCV RNA was quantified with the COBAS Amplicor HCV Monitor 2.0 assay (Roche Diagnostic Systems). A percutaneous liver biopsy was performed in accordance with the transplantation protocol and when clinically indicated. Hepatitis lesions were assessed according to the Metavir score.

HBV-DNA extraction

All the stored sera and liver samples from donor and recipient were analysed for HBV DNA.

DNA was isolated from serum with QIAamp UltraSens Virus Kit (Qiagen). Total DNA was extracted from 10 mg of liver sample with the MasterPure DNA purification kit (Epicentre) according to the manufacturer's instructions. DNA was eluted in 40 µl of water.

Detection of HBV DNA and direct sequencing

HBV DNA was detected using a highly sensitive PCR with primers located in the S and X genes as described previously.⁴ After electrophoresis PCR products were transferred by Southern-blot on nylon membrane and hybridized using an HBV probe labeled with ³²P-dCTP by terminal deoxynucleotide transferase (Boehringer Mannheim).

The amplified region was used for the determination of the patient's HBV genotype. Direct sequencing of the S gene was done using the purified PCR products and BigDye termination chemistry (Applied Biosystems) with an automated sequencer (ABIPrism). A phylogenetic analysis of all sequences was performed with sequences of different HBV genotypes.

HBV DNA quantification

To quantify HBV-DNA, a real time PCR-house assay performed on LC (Light Cycler, Roche Diagnostic Corporation, Germany) was performed using primers located in the core region of HBV: forward primer CSB (5'-TCG GAG TGT GGA TTC GCA CTC CTC-3', nucleotide position (nt) 2265-2288), and reverse primer CASB (5'-GAT TGA GAC CTT CCT CTG CGA GGC-3', nt 2322-2415). The lower detection limit of this assay (10 viral copies/ml) was evaluated against standards and expressed in genome copies/ml. The PCR reaction was carried out in a total volume of 20 µl containing 10 µl of DNA template, 2 µl of light cycler DNA master hybridization mixture (Taq DNA polymerase, reaction buffer, dNTP mixture and 10mM MgCl₂), 3.2 µl of 25mM MgCl₂, 0.3 µl each of the 20µM primers. Samples

were loaded into disposable capillaries, centrifuged, and placed in the light cycler whose program was as follows:

- 1) Denaturation of DNA and activation of the FastStart polymerase: 95°C for 10 min, slope 20°C/s.
- 2) 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 15 s. The programmed temperature transition rate was 20°C/s. Real-time PCR monitoring was achieved by measuring the fluorescence at the end of each cycle.
- 3) Melting curve: a single melt cycle was generated by holding the reaction at 95°C for 0 s, then at 65°C for 15 s, followed by slow heating at a transition rate of 0.1°C/s to 95°C.
- 4) Cooling: 40°C for 30 s with a slope of 20°C/s.

Monitoring of fluorescence occurred at regular intervals during the annealing phase and continuously throughout the melting phase. For each run, a standard curve was generated in a 5-log range (10 to 50000 copies/ml) by serial dilutions of the HBV-DNA biological standard from the Versant HBV-DNA kit (Bayer). The melting curve and quantitative analysis were conducted by using light cycler analysis software 3.5 following the manufacturer's instructions (Roche diagnostics applied science).

Amplification of complete HBV genomes, cloning and sequencing

For a complete characterization of the HBV genome, we use Rolling Circle Amplification (RCA)¹² that can amplify of full-length HBV genomes from samples with low HBV viral loads.

Complementation and ligation of the strand plus of HBV RC-DNA

Before DNA isolation, complementation of the plus-strand was performed by adding to 150 µl of serum 75 mL of 3 X reaction buffer (21.6 µl 0.8M Tris, pH ;10.8 µl MgCl₂, 0.8M; 10.8 µl NH₄Cl, 1.2M; 10.8 µl β-mercaptoethanol, 3%; 10.05 µl NP40, 20%; 2.2 µl of each dNTP, 10mM; sterile water to 75 µl). The reaction was performed for 12 hours at 37°C. After, the complementation product was treated with 10U of Rnase-Free DnaseI for 10 minutes at 37°C. HBV DNA was then isolated as described above for serum samples. To ligate the plus-strand of HBV RC-DNA, 8.5 µl of the extraction product were added to 1X ligation buffer and 1.5U T4 DNA ligase (Promega) in a final volume of 10 µl. The ligation reaction was carried out for 16 hours at 16°C, followed by heating at 65°C for 10 minutes to inactivate the ligase. Four microliters of this ligation product were used as DNA template for RCA.

Rolling circle amplification

Eight microliters of HBV DNA, isolated after complementation, were mixed with 8 phosphorothioate-modified primers (Table 2) at a concentration of 10 μ M each and 1X *Phi29* Buffer (New England Biolabs) in a final volume of 10 μ l. DNA mix was denatured at 95°C for 3 minutes, then cooled to room temperature in stages: 50°C for 15 seconds, 30°C for 15 seconds and 20°C for 10 minutes. Thereafter the denaturated product was placed on ice.

Table 2: Sequences and positions of the primers used for RCA.

Name	Sequence (15nt)	Position on HBV genome
RCA1	5'-AATCCTCACAAATA*C*C-3'	226-240
RCA2	5'-GATGGGATGGGAA*T*A-3'	615-601
RCA3	5'-CCTATGGGAGTGG*G*C-3'	637-652
RCA4	5'-GCAACGGGGTAAA*G*G-3'	1154-1140
RCA5	5'-ATGCAACTTTTTTC*A*C-3'	1814-1829
RCA6	5'-TCCAAATTCTTTA*T*A-3'	1916-1930
RCA7	5'-TAGAAGAAGAACT*C*C-3'	2374-2389
RCA8	5'-AGAATATGGTGAC*C*C-3'	2820-2834

*Indicate the thiophosphate linkages made on the two 3' terminal nucleotides of each primer to protect from exonuclease activity.

Sample mixtures were combined with 10 μ l of reaction mixture containing: 1X *Phi29* buffer, the 9 phosphorothioate-modified primers at a concentration of 10 μ M each, 0.4mg/ml of BSA, 2mM of dNTPs and 1 U/ μ l of *Phi29* DNA polymerase (New England Biolabs). Reactions were carried out at 30°C for 18 hours and terminated at 65°C for 10 minutes to inactivate the *Phi29* DNA polymerase.

RCA products analysis

Firstly 2 μ l of RCA products were run on a 0.8% agarose gel and stained with ethidium bromide. Secondly, a restriction analysis was made. One microliter of RCA product was digested in a final volume of 10 μ l with 3U of *SpeI* (New England Biolabs), cutting only one time in the HBV genome. Products of digestion were analyzed on a 1% agarose gel.

The RCA products were cloned into *Escherichia coli* by means of TA Cloning® Kit with pCR®2.1 (Invitrogen, France), and the clones were sequenced and analysed.

Immunohistochemistry

Deparaffinized tissue sections were labelled with anti-E2 monoclonal antibody for HCV as described previously.¹³

RESULTS

During the follow-up post-transplantation for HCV-related cirrhosis, the patient was HCV negative (RNA detection in the serum by RT PCR) and did not show any HBV marker except anti-HBc IgG and at one time-point positivity for HBsAg (Table 1). All biological parameters were normal but analysis of liver histopathology demonstrated an on-going chronic hepatitis with a low inflammatory activity process and mild fibrosis. However, the patient had no serological evidence of on-going hepatitis A, B, or C, cytomegalovirus or Epstein-Barr virus infections or other known diseases. Tests for antinuclear, anti-smooth muscle and anti-mitochondrial antibodies were also negative. To further investigate this chronic hepatitis of unknown aetiology, we looked for occult HBV using a sensitive in-house PCR.

The sera and liver samples of the liver donor, prior transplantation, were retrospectively analysed for HBV by nested PCR (HBV S and X genes) and by real time PCR (HBV C gene). No HBV DNA was found (data not shown).

We examined sections from liver biopsies of the explanted liver from the patient transplanted for HCV-related cirrhosis (anti-E2 staining), paired with section from liver draft from the donor. We observed a strong HCV staining of hepatocyte membranes, cytoplasmic and perinuclear regions in the explanted liver, while the liver donor was negative for anti-E2 staining (Figure 1). After transplantation, all period follow-up liver biopsies were negative for anti-HCV staining. These results correlate with the absence of HCV detection in serum during the long term follow-up of the patient after transplantation.

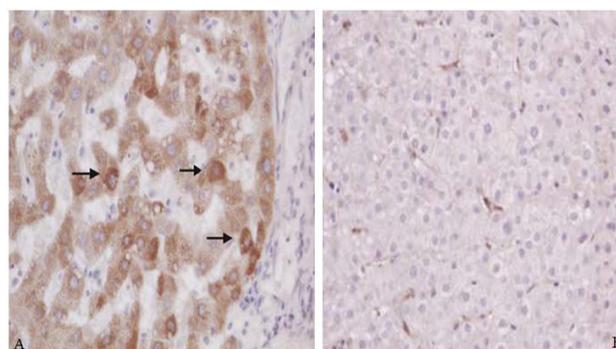


Figure 1: Immunostaining of liver sections (x200): A. Accumulation of E2 protein in liver of transplant recipient for HCV-related cirrhosis. B. Absence of anti E2 staining in donor liver.

All sera and liver samples of the transplanted patient, before and during the follow-up after transplantation, were retrospectively tested for HBV DNA. Using Nested PCR in the S and the X genes of HBV genome, HBV DNA was detected in all liver samples (pre-transplantation: 1994; post-transplantation: 1995, 1997 and 2000). In the sera, HBV DNA was detected in 5/11

post-transplantation samples in different HBV genes (4 times for the X gene, 3 for the S gene and 1 time for both). Results are shown in Table 3. HBV viral load quantified by real time PCR was low. Indeed, HBV DNA values ranged from 1.15×10^2 to 4.7×10^3 copies/mg in liver biopsies, while in sera, concentrations were between 85 and 2.8×10^3 HBV DNA copies/ml (Table 4).

Table 3: Serum HBV DNA in the recipient before and after transplantation.

Date	HBV region by PCR		HBV load (copies/ml)
	X gene	S gene	
04/ 1994*	+	+	85
05/ 1994	-	-	TND
08/ 1994	-	-	NT
11/ 1994	-	-	NT
01/ 1995	-	-	TND
03/ 1996	+	-	1.22×10^2
03/ 1997	+	+	2.8×10^3
01/ 1998	-	+	2.3×10^2
07/ 1999	-	-	TND
04/ 2000	+	-	1.1×10^2
09/ 2001	-	-	TND

*Serum before transplantation
NT: not tested; TND: target not detected

Table 4: HBV DNA in liver and histological score at time of analysis.

Date	HBV region by PCR		HBV load (copies/mg of liver)	Metavir score
	X gene	S gene		
1994*	+	+	3.2×10^2	A2F4*
1995	+	-	1.15×10^2	A1F1
1997	+	+	4.7×10^3	A1F1
2000	+	+	7.5×10^2	A0F1

*Native liver

All PCR products in the S gene from liver and sera were sequenced. The analysis of sequences showed a HBV strain genotype D. To assess whether or not the HBV sequences isolated from liver and sera before and after transplantation belong to the same strain, a phylogenetic analysis was performed on S gene sequences pre-transplantation and from sera and liver samples in 1997 that had the highest HBV load.

There was 96% homology between HBV pre-transplantation and post-transplantation liver and serum sequences. These results confirmed that there was endogenous reinfection of the new liver with occult HBV coming from the liver recipient.

To better understand if the occult HBV is correlated to HBV genome mutations, full length HBV genomes were amplified from sera using RCA and then sequenced. Analysis of the sequences confirmed that they were genotype D, and more specifically of subtype D3, but did not show mutations capable of inhibiting S gene expression and/or the viral replication that could explain the occult HBV status in this patient (Figure 2).

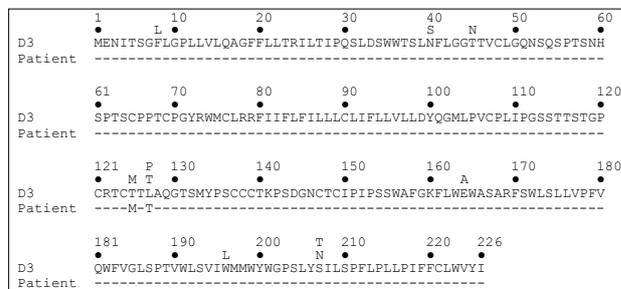


Figure 2: Alignment of the HBsAg sequence of the patient with a consensus genotype D3 HBsAg sequence.

An 80% consensus HBsAg sequence was constructed by aligning HBsAg sequences translated from 50 full-length genotype D3 genomes. At positions where no single residue was present in over 80% of the sequences, the most abundant residue at that position is placed in the consensus sequence and other residues present several times at that position are placed above the consensus sequence. -: Residue identical to the consensus sequence.

DISCUSSION

With the development of sensitive molecular tests for HBV in recent years, in various clinical situations low levels of HBV DNA have been detected despite the absence of HBsAg: (a) Transmission trough transfusion^{14,15} or transplantation^{16,17} (b) Viral reactivation in patients under condition of immunosuppression.^{18,19} (c) Chronic hepatitis of unknown aetiology,⁴ in HCC²⁰ and the highest prevalence in HCV patients.^{5,21} About 20–30% of sera and 40–50% of livers of anti-HCV positive patients with or without HCC demonstrated HBV DNA positivity.²⁰

Ghissetti and al. reported in 14 AgHBs patients undergoing liver transplantation, that 9 had occult HBV infection, among them, 7 patients were HCV positive.¹⁶ Some reports^{22,23} described that *de novo* post-transplantation HBV infection occurred at a high rate in recipients of donors with anti-HBc. In our case, the transplant recipient for HCV-related cirrhosis was anti-HBc positive while the donor did not have any HBV serological markers. In addition, histological examination of liver biopsies during the follow-up demonstrated a mild activity and portal fibrosis without septa whereas biochemical liver functions, including ALAT, ASAT, bilirubin and GGT, did not show significant abnormalities. Although some patients develop post-

transplantation recurrence of HCV viraemia without significant histologic injury, the progression of HCV is variable, with progression to cirrhosis in up to 30% of patients at 5 years.²⁴ Furthermore, Shiffman and al. have reported that in recurrent hepatitis C after orthotopic liver transplantation approximately 50% of patients had continually normal liver transaminases.²⁵ Thus, with our patient, HCV was extensively researched after liver transplantation in sera and liver samples using immunoassays, PCR and immunohistochemistry with anti-E2. All tests were negative for HCV.

Screening for hepatitis B serological markers showed an anti-HBc positive response in all samples while anti-HBs and HBsAg were undetectable excepted in one sample in 1997 where HBsAg was positive. In such situation, a temporary appearance of HBsAg in sera could be attributing to laboratory error. However, there are others explanations. The first is latent HBV infection that maintains low level replication, especially since the patient had anti-HBc serological markers indicating a past HBV infection. The second is *de novo* HBV infection. Both situations suggested the use of sensitive PCR for investigation of on-going HBV infection. In this study, to increase the sensitivity and the specificity, we used an in-house nested PCR with two different sets of primers located in the S and X genes of HBV,⁴ to analyse DNA extracts from liver as well as sera samples. HBV DNA was detected in sera and liver samples after transplantation and also in the liver and the sera of the recipient pre-transplantation but not in the liver donor. All livers samples from 3 available biopsies after transplantation were positive for HBV whereas in sera HBV DNA was found only 5/11 samples and more frequently using primers in the X than in the S gene. Several studies of occult HBV infections have reported that the frequency of HBV DNA was higher in liver than in sera.^{5,17,26} However, in some case of occult HBV infection, HBV DNA was positive in serum but not detected in the liver. Alternatively, other locations may support on-going HBV replication.^{27,28} Recent studies suggested recurrence of HBV infection both in positive or negative HBsAg patients after the removal of the infected liver and the implantation of the new, uninfected one.^{17,29} Abdelmaleck et al. reported that among 10 patients AgHBs negative but with detectable HBV DNA in liver before transplantation, 4 had HBV DNA in the liver post-transplantation without associated clinical hepatitis or HBsAg in sera and this was most likely to occur in patients who were co-infected with HCV.¹⁷ However, the authors of this study did not perform further analysis to assess whether the HBV infection found in the 4 cases was *de novo* infection or recurrence of occult HBV infection.

In our case, to determine the origin of HBV DNA, we sequenced PCR products in the gene S from sera and liver of our patient before and after transplantation. All sequences showed an HBV strain of genotype D. Phylogenetic analysis confirmed that the same HBV

strain was circulating in the liver recipient pre- and post-transplantation. Our findings demonstrated that liver transplant recipient for HCV-related cirrhosis with past exposure to HBV, but who tested negative for HBsAg, has transmitted an occult HBV infection leading to re-infection of the new grafted liver. Virological and clinical reactivations of occult or quiescent HBV infections have been observed in different clinical settings including hematological malignancies, HIV infection, hematopoietic stem cell transplantation, and organ transplantation.^{10,11,30} In the context of immunosuppression induced by therapies and/or related to diseases that involve the immune system, reactivation of occult HBV infections, despite the absence of HBsAg detection, seems to be an important phenomenon. In occult HBV infections, the mechanism leading to the presence of HBV DNA without HBsAg in some patients is still unclear. Some of these individuals are infected by viral variants either producing an antigenically modified HBV S protein undetectable by available HBsAg assays,³¹⁻³⁴ or carrying mutations capable of inhibiting the S gene expression and/or the viral replication reducing it to levels undetectable by many commercial assays.^{35,36}

In our case, after analysis of the full-length HBV genome, we confirmed that the strain was genotype D, but we did not find mutations that could explain suppression of viral replication or gene expression leading to the occult HBV status. However, the highest prevalence of occult HBV has been found in patients with hepatitis C virus infection and previous reports concerning *in vitro* studies have clearly demonstrated that the HCV “core” protein strongly inhibits HBV replication.³⁷⁻³⁹ This strong suppression of HBV activity is responsible not only for HBsAg negativity but also for the very low or even undetectable levels of serum HBV DNA characterizing most of the cases with occult infection.^{8,20,40} In our case, HBV titers in liver and sera were much lower than those usually found in HBsAg positive individuals. Furthermore, like in previous studies,⁴¹ we observed that HBV viraemia fluctuated both in sera and in liver samples. The mechanisms responsible for the inhibition of HBV replication and expression remain at present largely unclear. Many factors are involved, principally the host’s immune response which may play an important roles in inducing the occult status.

Considering the high frequency of occult HBV infections among HCV patients and its clinical impact in accelerating the progression of liver fibrosis to cirrhosis and hepatocarcinoma, it is important to monitor for occult HBV all patients undergoing immunosuppressive therapy.

The early identification of an HBV virological reactivation would prompt starting immediate specific antiviral therapy to prevent the occurrence of severe hepatitis B and possible liver transplantation failure.

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Ethical approval: The study was approved by the institutional ethics committee

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