Presence of Intrahepatic (Total and ccc) HBV DNA Is Not Predictive of HBV Recurrence After Liver Transplantation

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Previous studies reported that hepatitis B virus (HBV) deoxyribonucleic acid (DNA) can be detected in livers of patients who received transplants for hepatitis B despite the absence of serological markers of HBV recurrence. Quantification of HBV DNA was not performed and presence of covalently closed circular (ccc) DNA was not analyzed in most studies. We aimed to quantify total and ccc HBV DNA in explant liver and post-orthotopic liver transplantation (OLT) biopsies and to correlate the values with HBV recurrence post-OLT. Frozen liver tissue from 34 patients (9 with explant liver only, 9 with explant liver and post-OLT liver biopsies, and 16 with post-OLT biopsies only) in the National Institutes of Health HBV-OLT study was examined using real-time polymerase chain reaction (PCR). Among the 18 patients with explant liver, 7 were hepatitis B e antigen (HBeAg)-positive, 8 had detectable serum HBV DNA, and 10 received antiviral therapy prior to OLT. Total and ccc HBV DNA was detected in explant livers of 17 and 16 patients, respectively. Of the 10 patients who received antiviral therapy pre-OLT, serum HBV DNA was undetectable in 8 at transplantation but 7 had detectable total and ccc HBV DNA in their explant liver. Of the 25 patients with post-OLT biopsies, total HBV DNA was detected in 83% and ccc DNA in 17% of 47 biopsies, although only 2 patients had HBV recurrence. In conclusion, total and ccc HBV DNA could be detected in explant livers of most patients despite undetectable serum HBV DNA and hepatitis B surface antigen (HBsAg). Our findings suggest that occult HBV reinfection occurs in most HBV patients after OLT and continued administration of appropriate prophylactic therapy is important in preventing overt HBV recurrence. Liver Transpl 13:1137-1144, 2007. © 2007 AASLD.

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Chronic hepatitis B virus (HBV) infection is maintained by a replicative form of HBV deoxyribonucleic acid (DNA), the covalently closed circular (ccc) DNA. HBV ccc DNA persists in the hepatocyte nuclei as a stable episome and acts as a template for the transcription of viral genes. Based on studies in ducks, it has been estimated that ccc DNA is present at a concentration of 30 to 50 copies per cell.1,2

Previous studies reported that interferon and nucleoside analogs such as lamivudine were ineffective in decreasing hepatic ccc DNA. Recent studies using more

Abbreviations: HBV, hepatitis B virus; OLT, orthotopic liver transplantation; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; ccc, covalently closed circular; HBeAg, hepatitis B e antigen; HBIG, hepatitis B immune globulin; HBsAg, hepatitis B surface antigen.

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sensitive techniques showed that both pegylated interferon and adefovir can decrease ccc DNA but the effect on ccc DNA was less marked than that on serum HBV DNA or hepatic total HBV DNA.\textsuperscript{3-7} One study also found that ccc DNA remained detectable in persons who had recovered from past HBV infection.\textsuperscript{6} The persistence of ccc DNA accounts for the high frequency of viral relapse when antiviral therapy is stopped, prompting the suggestion that monitoring of hepatic ccc DNA should be used to assess treatment response and to determine when antiviral therapy can be discontinued.

Use of hepatitis B immune globulin (HBIG) and/or nucleoside/tide analogs in the past 20 yr has decreased the rate of HBV reinfection post-liver transplantation from 80\% to approximately 10\%.\textsuperscript{8} However, several studies reported that HBV DNA can be detected in the liver and peripheral blood mononuclear cells of patients with no serologic evidence of HBV reinfection post-orthotopic liver transplantation (OLT), indicating that HBV prophylaxis must be maintained for life.\textsuperscript{9,10} These studies did not examine ccc DNA, and hepatic HBV DNA analysis was qualitative. In addition, a correlation between the detection of hepatic HBV DNA and HBV recurrence had not been examined.

We aimed to quantify intrahepatic HBV DNA (total and ccc) in the explant liver and to correlate with: 1) HBeAg status and serum HBV DNA at transplant; 2) antiviral therapy pre-OLT; and 3) HBV recurrence post-OLT. We also aimed to determine the rate of HBV DNA detection in the post-OLT liver and to determine if HBV DNA detection in the post-OLT liver is a predictor of HBV recurrence.

**PATIENTS AND METHODS**

**Patients**

Stored liver tissues from 34 patients enrolled in the National Institutes of Health sponsored study on “Prevention of Recurrent Hepatitis B Post-Liver Transplantation” were studied (9 with explant liver only, 9 with explant liver and post-OLT biopsies, and 16 with post-OLT biopsies only). This study is a retrospective-prospective observational study that enrolled consecutive hepatitis B surface antigen (HBsAg)-positive patients >13-yr-old from 15 centers in the United States, who were listed for liver transplantation between November 2001 and June 2005.\textsuperscript{11} The use of human liver tissue was approved by the Institutional Review Boards at each of the participating centers and written consent was obtained from all patients. Liver samples were snap frozen in liquid nitrogen and stored at −80°C prior to testing. Samples were shipped on dry ice from participating centers to the central laboratory at the University of Michigan.

**Definitions**

HBV recurrence was defined as the presence of serum HBsAg and/or serum HBV DNA levels greater than 5 log\textsubscript{10} copies/mL on 2 consecutive occasions at least 1 month apart. Virologic breakthrough was defined as an increase in serum HBV DNA by >1 log\textsubscript{10} copies/mL compared to nadir during antiviral therapy. Genotypic resistance was defined as detection of antiviral-resistant mutations: rtM204I/V ÷ rtL180M for Lamivudine (methionine to isoleucine or valine substitution with or without leucine to methionine substitution), and rtA181V/T (alanine to valine or threonine substitution) and/or rtN236T (asparagine to threonine substitution) for Adefovir.

**Serum HBV Markers**

All HBV serology tests pre- and post-OLT were performed locally using commercially available assays at the participating centers.

Serum HBV DNA levels were quantified by the Cobas Amplicor HBV Monitor assay (Roche Molecular Systems, Branchburg, NJ) at the central laboratory in the University of Michigan. The lower limit of detection was 200 copies/mL. Samples with HBV DNA >200,000 copies/mL were retested after 1 in 1,000 dilution.

**Quantification of Intrahepatic HBV DNA**

**DNA Extraction From Liver Tissue**

HBV DNA was extracted from 25 mg of liver explants or 5-ng to 25-mg post-OLT biopsies using the QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The DNA was eluted in 100 \mu L of sterile double-distilled water. A 50 \mu L aliquot was put aside for quantification of total HBV DNA and \beta-actin DNA. The remaining 50 \mu L was treated with 0.25 units of Plasmid-Safe adenosine triphosphate-dependent DNase (Epigen Technologies, Madison, WI) for 30 minutes at 37°C to digest the relaxed circular and single-stranded HBV DNA. The DNase enzyme was inactivated by incubating at 70°C for 30 minutes. The enzyme-treated DNA was further purified using the QIAquick polymerase chain reaction (PCR) purification kit (Qiagen) according to the manufacturer’s instructions. The DNA was then eluted in 50 \mu L of sterile double-distilled water. A total of 10 \mu L of DNase-treated DNA was used for the quantification of ccc DNA, while 10-\mu L of untreated DNA each was used for the quantification of total HBV DNA and of \beta-actin DNA using real-time PCR.

**Real-Time PCR for Quantification of ccc and Total Intrahepatic HBV DNA**

Conserved regions in the HBV genome were identified by aligning almost 50 full-length sequences corresponding to HBV genotypes A-H obtained from GenBank using the MEGALIGN program (DNASTAR, Madison, WI). Primers and probes for ccc DNA amplification targeted across the single-stranded gap region of relaxed circular HBV DNA and primers and probes for total HBV DNA amplification targeted to a double-stranded region of HBV DNA were designed using the PrimerSelect software (DNASTAR). All primers and probes are listed in Figure 1 and were synthesized by...
Integrated DNA Technologies (Coralville, IA). Real-time PCR was performed in an iCycler iQ Multi-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) as described previously. To maintain consistency between different PCR assays, serial dilutions of the pAM6 plasmid and human genomic DNA standards were prepared in bulk and stored frozen in aliquots to avoid freeze/thaw cycles and to increase precision. To monitor for contamination during each step, sterile double-distilled water and liver specimens from uninfected patients (HBsAg and hepatitis B core antibody –negative with undetectable HBV DNA in serum by PCR assay) were used as negative controls.

Calibration of the PCR Assay and Determination of Sensitivity and Range of Linearity

The plasmid DNA construct pAM6 (ATCC, Manassas, VA) containing a full-length HBV monomer served as the standard for total and ccc DNA quantification. Serial dilutions of the plasmid pAM6 were tested along with serial dilutions of plasmid HBV-A (an HBV genotype A plasmid that had been previously calibrated against the World Health Organization HBV standard). The amplification efficiency of the plasmid pAM6 was the same as that of the plasmid HBV-A as seen by the identical slope of the superimposed calibration curves (data not shown). Testing in duplicates on 2 occasions yielded consistent results. A linear relationship was observed over the range of plasmid concentration from $2.5 \times 10^4$ to $10^{10}$ copies/mL.

Sensitivity

To determine the lower limit of detection, liver tissue from 2 liver donors who were seronegative for HBsAg and hepatitis B core antibody, with undetectable HBV DNA in serum was spiked with plasmid pAM6 at concentrations 5, 10, 25, 50, 100, 200 and 1,000 copies/mL, followed by DNA extraction and enzymatic treatment on 4 different occasions. Each DNA extract was tested by real-time PCR in duplicates using ccc and total DNA primers, in 3 different assays, as described above. Plasmid DNA concentrations greater than 25 copies/mL were consistently detected in all assays, while concentrations between 10 and 25 copies/mL were detected in 30% of the assays.

Specificity and Efficacy of Adenosine Triphosphate DNase Enzyme

To determine the specificity of the ccc DNA detection, HBV DNA extracted from serum of 6 HBV infected patients with median HBV DNA levels of $3.35 \times 10^4$ copies/mL (range, $1.21 \times 10^3$ to $3.5 \times 10^8$) were amplified using ccc and total HBV DNA primers. HBV DNA was detectable with total primers but was undetectable with ccc primers in all 6 patients.

To evaluate the efficacy of the DNase enzyme in digesting relaxed circular and single stranded forms of HBV DNA, DNA extracted from serum and liver samples with and without DNase treatment were amplified using primers for detection of total and ccc HBV DNA. Using total primers, HBV DNA was detected in all 6 untreated but in none of the DNase treated serum samples confirming that the DNase degraded relaxed circular forms of HBV. Total HBV DNA was amplified in all 6 liver samples regardless of DNase treatment while ccc DNA concentrations from enzyme treated samples were a mean of 1.2 log lower than those from undigested liver samples.

Reproducibility

To determine the reproducibility of our assay, DNA was extracted from explant liver samples on 2 to 4 different occasions. For post-OLT biopsies, DNA was extracted on 1 to 2 separate occasions depending on the size of the biopsies, and each DNA extract was tested in duplicates on 2 separate occasions. The mean coefficient of variation for intraassay and interassay variability was 0.063 and 0.098, respectively.

Real-Time PCR for Quantification of β-Actin

Quantification of β-actin was used to estimate the amount of genomic DNA in each liver sample. β-Actin amplification was performed using real-time PCR as described above using the TaqMan β-actin control kit (PE Applied Biosystems, Foster City, CA). Serial dilutions of human genomic DNA served as standard for β-actin quantification. Using the formula of 6.667 pg of

Figure 1. Positions and sequences of the primers and probes used for real-time PCR.
human genomic DNA per cell, the number of cells in each liver sample was estimated and the amount of HBV DNA per cell was calculated. The detection limits of our hepatic HBV DNA assays were estimated to be $2 \times 10^{-6}$ and $1 \times 10^{-6}$ copies/cell for ccc and total HBV DNA, respectively.

Statistical Analysis

Statistical analyses were performed by SPSS software (version 13.0; SPSS, Chicago, IL). Continuous variables were expressed as median (range) unless otherwise indicated, and compared using Mann-Whitney U-test. Categorical variables were compared by chi-squared test. HBV DNA was logarithmically transformed for analysis. Assay reproducibility was assessed by the coefficient of variation. The correlation of serum HBV DNA, intrahepatic total HBV DNA, and ccc DNA was analyzed by Pearson correlation test. Statistical significance was taken as $P$ value less than 0.05. All statistical tests were 2-sided.

RESULTS

Analysis of Explant Liver HBV DNA

Correlation With HBeAg Status, Serum HBV DNA, and Antiviral Therapy at Transplantation

Among the 18 patients with explant liver, 7 were HBeAg-positive, 10 were HBeAg-negative, and HBeAg status was unknown in 1 patient. Characteristics of the patients are listed in Table 1. Serum HBV DNA was detectable in 8 (50%) patients with a median HBV DNA level of $\frac{6.07}{10}$ log copies/mL. Ten patients had hepatocellular carcinoma.

Total and ccc HBV DNA were detected in 17 (94%) and 16 (89%) patients, respectively, with median HBV DNA levels of $-1.39$ and $-2.68 \log_{10}$ copies/mL, respectively. HBeAg-positive patients had higher concentrations of ccc DNA ($-1.39$ vs. $-3.08 \log_{10}$ copies/cell, $P = 0.037$) and higher ratios of ccc/total HBV DNA ($7.9\%$ vs. $2.7\%$, $P = 0.046$) in patients without recurrence. Concentrations of total and ccc HBV DNA in explant liver were slightly higher in these 2 patients than patients with no HBV recurrence, but the difference was not significant. Neither patient with HBV recurrence received any antiviral therapy pre-OLT.

Analysis of Post-OLT liver HBV DNA:

A total of 25 patients with 47 biopsies collected between 7 days to 48 months post-OLT were studied. Characteristics of these 25 patients are summarized in Table 2. Eight biopsies had detectable total and ccc DNA, 31 had detectable total but not ccc DNA, and 8 had no detectable total or ccc DNA. In the 39 samples with detectable total HBV DNA, the median concentration of total HBV DNA was $-4.59 \log_{10}$ copies/cell. The concentration of total HBV DNA was higher in the 8 samples with detectable ccc DNA, median $-3.33$ vs. $-4.97 \log_{10}$ copies/cell in the 31 samples with detectable total but not ccc HBV DNA, ($P = 0.007$). The median concentration of ccc DNA in those 8 samples was $-4.83 \log_{10}$ copies/cell.

Three patients received liver grafts from donors who were positive for hepatitis B core antibody. Total HBV DNA was detected in all 8 post-OLT biopsies from these 3 patients but ccc DNA was detected in only 1 of the biopsies.

Seven patients had detectable total (12/12 biopsies) and ccc (8/12 biopsies) HBV DNA in their biopsies; of these, 1 patient had HBV recurrence after 7 months of post-OLT follow-up (patient 3). A total of 14 patients had detectable total (27/30 biopsies) but not ccc (0/30 biopsies) HBV DNA; of these, 1 patient had HBV recurrence after 10 months of post-OLT follow-up (patient 2). Four patients had undetectable total and ccc HBV DNA in all their biopsies (0/5), none had HBV recurrence (Fig. 3).

As shown in Figure 4, total HBV DNA was detected in 83% post-OLT biopsies while ccc DNA was detected in 17% of the post-OLT biopsies from patients who did not meet criteria for HBV recurrence throughout the duration of post-OLT follow-up. Figure 5 shows that among patients who had explant liver and post-OLT biopsies studied, concentrations of total HBV DNA in the first biopsy obtained post-OLT decreased by 2 to 5 log compared to the explant liver. However, concentrations of total HBV DNA remained stable over time among 15 patients with no HBV recurrence in whom serial post-OLT biopsies were tested.
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**Abbreviations:** M, male; F, female; UD, undetectable; NA, not available; Pos, positive; Neg, negative; Lam, lamivudine; Adv, adefovir; W, White; A, Asian American; AA, African American.

*Patients 3 and 9 had HBV recurrence after 1 and 10 months post-OLT, respectively.

†Patient 4 had breakthrough while on Lam.
Clinical Course of the Patients With HBV Recurrence

In total, 3 patients had HBV recurrence.

Patient 1 had serum HBV DNA level of 8.54 log_{10} copies/mL pre-OLT but did not receive antiviral therapy. The explant liver had detectable total and ccc HBV DNA levels of −0.62 and −2.57 log_{10} copies/cell, respectively. Intravenous HBIG 10,000 IU was adminis-
tered during the anhepatic phase, daily during the first 3 days, and then monthly. This patient became HBsAg-positive with serum HBV DNA level of 4.09 log_{10} copies/mL 1 month post-OLT. Post-OLT biopsies were not available for intrahepatic HBV DNA analysis. Lamivudine was initiated after diagnosis of HBV recurrence.

Patient 2 had serum HBV DNA level of 9.04 log_{10} copies/mL pre-OLT but did not receive antiviral therapy. The explant liver had very high concentrations of
total and ccc HBV DNA: 1.52 and −0.24 log_{10} copies/cell, respectively. Lamivudine was started immediately post-OLT and intravenous HBG 10,000 IU was administered during the anhepatic phase, daily during the first week, and then monthly. This patient became HBsAg-positive 10 months post-OLT. However, serum HBV DNA remained undetectable despite persistent detection of serum HBsAg. Total HBV DNA but not ccc DNA was detected in liver biopsies obtained at 3 and 12 months post-OLT.

Patient 3 received lamivudine for 2 yr prior to OLT. Serum HBV DNA just before OLT was 8.62 log_{10} copies/mL. The explant liver was not available for study. Post-OLT, the patient received combination of lamivudine and intravenous HBG 10,000 IU during the anhepatic phase, daily during the first 10 days, and then every 1 to 2 weeks. Despite the frequent high doses of HBIG, this patient became HBsAg-positive with detectable serum HBV DNA at 3 months post-OLT. Resistance to lamivudine (rtL180M, rtM204V) was confirmed and adefovir was added. Total and ccc HBV DNA were detectable in the biopsy obtained at month 4 post-OLT in concentrations of 1.94 and 0.04 log_{10} copies/cell.

**DISCUSSION**

In this study, we found that almost all hepatitis B patients had detectable total and ccc HBV DNA in their explant liver although 56% of these patients had received antiviral therapy pre-OLT and 56% had undetectable serum HBV DNA at transplant. Our data are in accordance with that of other investigators, indicating that while antiviral therapy can decrease hepatic HBV DNA including ccc DNA, the effect is substantially less than that on serum HBV DNA. Mathematical modeling suggests that continued treatment for at least 10 yr using an antiviral agent with little or no resistance is required to achieve viral clearance. These findings account for the high frequency of viral relapse when antiviral treatment is stopped. Although the number of patients studied was small, it is worth noting that serum HBV DNA level at transplant was a better predictor of HBV recurrence than hepatic HBV DNA. This may be related to the fact that the native liver is discarded at transplant, and HBV in the circulation and extrahepatic reservoirs serve as the source of reinfection.

Our study demonstrated that total HBV DNA can be detected in the post-OLT biopsies of most patients supporting the concept that HBV reinfection occurs in most patients after liver transplantation and that overt manifestations of HBV infection are kept in check by continued administration of antiviral therapy and/or HBIG. Based on the high rate of detection of total HBV DNA in liver biopsies obtained within the first 6 months of OLT, we surmise that reinfection occur very early post-OLT and persists in most patients. Our data suggest that the presence of HBV DNA in post-OLT liver biopsies is not necessarily a harbinger for overt HBV recurrence, as long as appropriate HBV prophylaxis is maintained. Of the 2 patients who had HBV recurrence and had post-OLT biopsies studied, 1 patient had a high serum HBV DNA level and did not receive antiviral therapy pre-OLT, while the other had lamivudine breakthrough just before OLT and did not receive additional adefovir therapy post-OLT. In the remaining 23 patients, 19 had detectable total HBV DNA in at least 1
post-OLT biopsy with no evidence of HBV recurrence, up to 46 (median 7) months from the last available biopsy with detectable total HBV DNA. In addition to total HBV DNA, we also detected ccc DNA in 17% of the post-OLT biopsies. The lower rate of ccc DNA detection compared to total HBV DNA is likely related to a lower concentration of ccc DNA.

The sensitivity of our assay for total and ccc DNA was comparable to that reported by other investigators. We adopted the same measures described by Werle-Lapostolle et al. to verify the specificity of our assay for ccc DNA and were unable to detect ccc DNA in any of the 6 serum samples tested. Our finding of higher concentrations of total and ccc HBV DNA and a higher ratio of ccc:total HBV DNA in the explant liver of HBeAg-positive patients is also in keeping with that of previous reports.

In summary, total and ccc HBV DNA could be detected in the explant livers of most patients with hepatitis B, including those who had undetectable HBV DNA in serum. The concentration of ccc DNA and the ratio of ccc to total intrahepatic HBV DNA were higher in HBeAg-positive patients than in HBeAg-negative patients. Total but not ccc HBV DNA could be detected in post-OLT liver biopsies of most patients despite undetectable serum HBV DNA and HBsAg. Our findings suggest that occult HBV reinfestation occurs in most HBV patients after liver transplantation and continued administration of appropriate prophylactic therapy is important in preventing overt HBV recurrence. Validation of our data by other investigators is important and will determine if HBIG withdrawal and maintenance with nucleoside analogues alone will be sufficient in the long-term prevention of HBV recurrence after liver transplant.

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