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BRIEF REPORT

Quantification of Viral RNA in Multiple Pieces of Explant Liver Tissue Shows Distinct Focal Differences in Hepatitis B Infection

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Hepatitis B virus (HBV) DNA and RNA were quantified by digital PCR assays in 20–30 tissue pieces from each of 4 liver explants with cirrhosis caused by HBV. The within-patient variability of HBV RNA levels between pieces was up to a 1000-fold. Core RNA and S RNA levels were similar and correlated strongly when replication was high, supporting that transcription was from covalently closed circular DNA (cccDNA). By contrast, enhanced expression of S RNA relative to cccDNA and core RNA in patients with medium-high or low replication supports that HBV surface antigen (HBsAg) can be expressed mainly from integrated HBV DNA in such patients.

Keywords. droplet digital PCR; hepatitis B virus; HBsAg; cirrhosis; hepatocellular carcinoma.

Chronic infection with hepatitis B virus (HBV) is an important cause of liver cirrhosis and hepatocellular carcinoma (HCC) worldwide [1]. Viral clearance involves T-cell mediated eradication of infected cells, which reduces the number of episomal templates (covalently closed circular DNA, cccDNA) available for HBV replication. Additional mechanisms, including suppressed transcription of pregenomic RNA from cccDNA, contribute to the reduction of viral replication [2–4].

HBV surface antigen (HBsAg) is embedded in the envelope of viral particles and in the nucleic acid free subviral particles. Loss of HBsAg from the blood defines clinical resolution of HBV infection and lowers the risk for HCC development [5]. HBsAg in blood, however, often remains at high levels also when viral replication has ceased, and it has been hypothesized that this may be due to expression of HBsAg from HBV DNA integrated into the human genome [6, 7]. Integrated HBV DNA is replication incompetent because the core RNA, which serves as pregenomic RNA and is reversibly transcribed into the DNA genome of the virus, spans more than 1 round of the circular genome. Integrated HBV DNA allows expression of HBsAg, but not core antigen because the typical linearization at nt 1820 separates the core gene from its promotor that normally initiates transcription of the gene from cccDNA [7–11]. To clear HBsAg from serum, it is probably required that T cells recognize and eradicate also hepatocytes that produce HBsAg only from integrated HBV DNA. However, the immunological mechanisms involved in clearance of hepatocytes are largely unknown.

We have recently shown that a large fraction of the intrahepatic DNA is already integrated in the early HBeAg-positive stage of infection. The fraction of integrated DNA increased with disease progression, suggesting that integrated DNA is cleared less efficiently than replicating viral DNA [12]. The mechanisms involved in eradication of cells that carry only integrated HBV DNA as compared to cells that contain cccDNA, alone or in combination with integrated HBV DNA, are poorly known.

We recently used deep sequencing to analyze human and viral RNA in explant tissue from patients with HBV-related end-stage liver disease [13], and droplet digital polymerase chain reaction (ddPCR) to quantify different HBV RNA species in liver biopsies from patients in different stages of chronic HBV infection [14]. The findings in both studies support that a large proportion of HBV RNA in liver tissue originates from expression of integrated HBV DNA. In the present study, we explore this further by quantifying HBV RNA and DNA in multiple explant tissue pieces.

METHODS

Patients and Samples

The 4 patients included in this study underwent liver transplantation because of HBV-induced liver cirrhosis, 1 patient also having HCC (details in Supplementary Material).

HBV Serum Markers

HBV DNA levels in serum were analyzed by the Cobas Taqman or Cobas 6800 assays (Roche Diagnostics), and HBsAg levels in serum by the Architect assay (Abbott).

Nucleic Acid Extraction

Liver pieces 2–5 mm in size, frozen at −80°C were homogenized and nucleic acids were extracted using the MagNA Lyser

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and MagNA Pure instruments together with the MagNA Pure LC DNA Isolation Kit II according to the instructions (Roche Diagnostics). A portion of the extraction was DNAse treated with TURBO DNA-free kit (Thermo Fisher Scientific).

Droplet Digital PCR

The ddPCR analyses were carried out in duplicate reactions, containing primers (750 nM), probe (500 nM), with 5 µL of extracted nucleic acid and either (for quantifying DNA) 1× ddPCR supermix for probes (Bio-Rad), or (for quantifying RNA) One-Step RT-PCR mix (Bio-Rad). After rigorous mixing, droplets were generated in an AutoDG Automated Droplet Generator (Bio-Rad) with oil for probes (Bio-Rad). The amplification was performed on a Veriti Thermocycler (Applied Biosystems) using a thermal profiling with 48°C for 2 minutes, 95°C for 10 minutes for enzyme activation followed by for 40 cycles at 95°C for 30 seconds, 58°C for 1 minute, and 78°C for 1 minute (for cccDNA amplification only), 1 cycle at 98°C for 10 minutes to deactivate the enzyme, ending at 4°C. For RNA, an initial reverse transcription step for 60 minutes at 48°C preceded the cycling. After overnight incubation at 4°C, the plate was analyzed in a QX200 Droplet Reader (Bio-Rad) and QuantaSoft (Bio-Rad) software. A negative template control and a positive control were included in each run. Reactions with fewer than 5 positive droplets were considered to be under the detection limit and were excluded from analysis. The primers and probes used are presented in the Supplementary Material and Figure 1A. Quantifications of HBV RNA included a reaction without the reverse transcriptase to verify the efficiency of DNAse treatment.

Ethics

The principles of the Declaration of Helsinki were followed. The Regional Ethical Review Board in Gothenburg approved the study. All participants gave written informed consent.

Statistics

The degree of focality was measured as the proportion of pieces with detected target, and as the levels, as well as standard deviation (SD) and coefficient of variation (CV) for linear values, of HBV DNA and RNA in the 20–30 pieces. Correlations between levels of different HBV RNA and DNA species were analyzed by regression and variance analysis.

RESULTS

Assay Performance

To test the reproducibility of the whole procedure (extraction and ddPCR) 2 pieces from patient 3 were used. Five replicates from each piece were extracted and analyzed using ddPCR for S DNA. As shown in Figure 1A, right panel, the coefficient of variation, CV, was 10% when the observed concentration was 76–101 copies per 1000 cells. The cell count was determined by ddPCR of the human gene β-globin (Supplementary Material).

HBV Infection Is Unevenly Distributed

As shown in Figure 1B–1D, significant differences in viral RNA and cccDNA content between pieces were observed in all patients. Patient 1 (acute-on-chronic hepatitis with high HBV DNA in serum despite entecavir treatment for 3 weeks) and patient 2 (no effective antiviral treatment) had high or relatively high levels of both HBV DNA and RNA in all tissue pieces, with up to 10-fold difference in copies per 1000 cells between pieces (Figure 1 and Supplementary Material). Patient 3 (on tenofovir for 4 months prior to transplantation) had lower and highly diverging levels of core and S RNA, with up to a 1000-fold difference between pieces. As shown in Figure 1A, the HBV transcripts share a common 3′ end. Therefore, the S RNA ddPCR system detects both core RNA and S RNA. Patient 4 (on tenofovir since at least 2 years before transplantation) had undetected HBV DNA in 20%, and undetected core RNA in 65%, of the pieces.

Present or Absent Correlation Between S RNA and Other Markers

The levels of intrahepatic HBV DNA were highest in patient 1, whose HBV DNA was approximately 100 times higher than in patient 3, and 10 000 times higher than in patient 4. S RNA and core RNA correlated strongly in patient 1 and patient 3 (Figure 2A). In patient 2 and patient 4, higher proportions of S RNA in comparison to core RNA were observed, and no correlation was observed between these 2 RNAs.

The levels of cccDNA differed markedly between the patients, from approximately 2 copies of cccDNA per cell in patient 1 to approximately 1 copy of cccDNA per 100 cells in patients 2 and 3 (Figure 2B and Supplementary Material). No cccDNA was detected in the pieces from patient 4. As shown in Figure 2B, core RNA levels in the different tissue pieces correlated with cccDNA. This rather close correlation with cccDNA argues that the rate of core RNA transcription was similar in most of the samples. As shown in Figure 2C, S RNA correlated with cccDNA in patients 1 and 3. In patient 1, both core and S RNA were at levels around 100 copies per cccDNA with little variation. By contrast, in patient 2, S RNA did not correlate with cccDNA, and the S RNA copy numbers per cccDNA showed a pronounced variation between the pieces and was much higher than core RNA per cccDNA. A plot of the S RNA/core RNA ratio versus cccDNA shows that patient 3 had values in between patient 1 and patient 2 (Figure 2D). Notably, 4 pieces from patient 3 had ratios that were comparable to the ratios observed in patient 2. Furthermore, even though core and S RNA correlated in patient 3, the S RNA/core RNA ratio was 3-fold higher in patient 3 than in patient 1. In addition, the CV of the ratio was higher in patient 3 than 1 (174% vs 14%), even when the 4 pieces with the highest ratio were omitted

Figure 1. *A*, Positions of the ddPCR target regions in relation to the various HBV RNA species and open reading frames, and its performance, exemplified by analysis of S DNA levels in 5 different extractions of 2 liver tissue pieces from patient 3, 1 with low (piece 9) and the other with very low HBV DNA level (piece 3). *B–D*, The levels observed in multiple liver tissue pieces from each patient by ddPCR targeting S RNA (total HBV RNA) (*B*), core RNA (*C*), and cccDNA (*D*) expressed as copies/1000 cells. The number of cells was determined using ddPCR targeting the β-globin gene. Abbreviations: cccDNA, covalently closed circular DNA; ddPCR, droplet digital PCR; HBV, hepatitis B virus; Pat, patient.

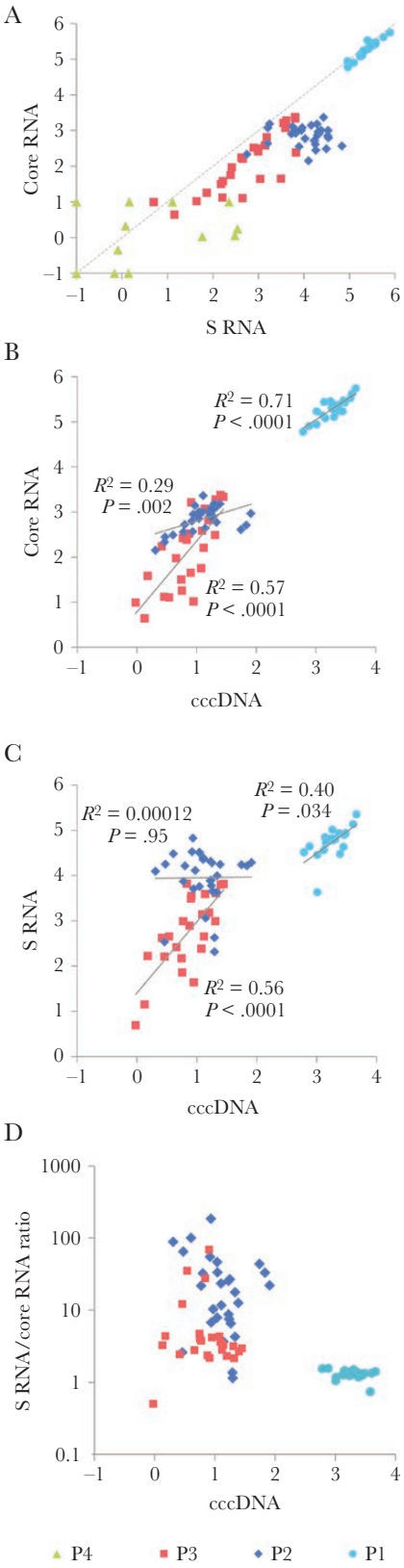

Figure 2. *A*, Correlations between core RNA and S RNA (total HBV RNA) in 4 HBV patients (patients 1–4). Twenty to thirty pieces from each patient were analyzed by ddPCR. In theory, if core RNA was 50% of the total HBV RNA it would be 0.3 $\log_{10}$ units below the dotted line. *B* and *C*, Correlation between levels of cccDNA and core RNA (*B*), and cccDNA and S RNA (*C*) in 20–30 pieces from each of 3 patients (patients 1–3). In patient 2, there was no association between cccDNA and S RNA. Levels are shown as $\log_{10}$ copies/1000 cells. *D*, Log_{10} copies cccDNA/1000 cells vs the ratio between S RNA and core RNA. Abbreviations: cccDNA, covalently closed circular DNA; ddPCR, droplet digital PCR; HBV, hepatitis B virus.

(31% vs 14%). These observations might indicate that also in patient 3 there was considerable S expression from integrated HBV DNA.

DISCUSSION

This study provides novel data regarding the distribution of HBV in the infected liver and correlations between different forms of HBV DNA and RNA. A total of 475 ddPCR analyses were performed on 95 liver samples from 4 patients. These patients represented different degrees of HBV infection: 1 highly active infection with approximately 2 cccDNA copies/cell, 2 moderately active with approximately 1 cccDNA copy/100 cells, and 1 with low active infection and no detected cccDNA.

In patient 1, with highly active infection, the HBV RNA levels were high in all tissue pieces with little variation, similar levels of core RNA and S RNA, and strong correlation between these RNA species. This finding indicates that core RNA and S RNA were both transcribed from cccDNA and at similar rates (resulting in the presence of approximately 100 copies of each transcript per cccDNA). Patient 2 was strikingly different. The levels of S RNA were much higher than core RNA, and there was no correlation between core RNA and S RNA or between cccDNA and S RNA. Similarly, in patient 4, with very low viral activity, there was more transcription of S RNA than core RNA and no correlation between these RNA species; cccDNA was not detected in any piece.

The S RNA levels per cccDNA were up to 50 times higher in patient 2 than in patient 1. To our knowledge, there is no known mechanism that could explain such an enhanced expression of S RNA from cccDNA or the very wide range of S RNA/cccDNA ratios that we observed. Such variation would, however, be expected if S RNA was expressed from HBV DNA integrations that were present in different numbers in different pieces. Thus, the findings in both patients 2 and 4 suggest that HBsAg likely was expressed mainly from integrated HBV DNA.

In patient 3, with moderately active infection, core RNA and S RNA also correlated strongly. However, 4 pieces had similar S RNA to core RNA ratios as the pieces from patient 2. Furthermore, the mean ratio and the CV of the ratios were higher than in patient 1, indicating that a considerable part of the S RNA also in patient 3 could originate from integrated HBV DNA.

These results extend previous comparisons of the HBV RNA expression pattern between different patients [13, 14] and chimpanzees [15] within-patient comparisons of the correlation between different HBV RNA forms.

The antiviral treatment and presence of liver cirrhosis in our patients are limitations of the study, as the results might not be

representative for HBV infections in general. However, nucleoside analogues block reverse transcription and do not directly influence the production of RNA. The presence of liver cirrhosis should not itself influence transcription from cccDNA, but might be associated with relatively higher levels of S RNA if associated with a higher proportion of integrated HBV DNA. In addition to S RNA, X RNA can be expressed from integrated HBV DNA. However, because ddPCR assays targeting S and X gave very similar results in our previous study of biopsies [14] we have focused on S RNA in the current study.

In summary, our investigation demonstrates large differences in the distribution of HBV infection, in particular when viral load is low, supports that a large proportion of intrahepatic HBV DNA is integrated in the human genome, and suggests that HBsAg may be expressed from these integrations. The results also demonstrate high performance and utility of digital PCR and encourage further HBV research on explanted livers.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Author contributions. All authors have contributed to, seen, and approved the final, submitted version of the manuscript.

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