Abundance of non-circular intrahepatic hepatitis B virus DNA may reflect frequent integration into human DNA in chronically infected patients

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Key points: Quantification of circular and linear HBV DNA in liver biopsies from patients with chronic infections suggested that a large fraction of the DNA was integrated into the host genome, which has implications for disease clearance markers and hepatocellular carcinoma development.
ABSTRACT

Background: Hepatitis B virus (HBV) integration has implications for cancer development and surface antigen (HBsAg) production, but methods to quantify integrations are lacking. The aim of this study was to develop a digital PCR (ddPCR) assay discriminating between circular and integrated HBV DNA, and to relate the distribution between the two forms to other HBV markers. Methods: ddPCR comprising primers spanning the typical linearization breakpoint in the HBV genome allowed for quantification of the absolute copy numbers of total and circular HBV DNA, and calculation of linear HBV DNA. Results: Analysis of 70 liver biopsies from patients with chronic HBV infection revealed that the fraction of linear HBV DNA, which includes integrations, was higher in HBeAg-negative patients than HBeAg-positive. The ratio between HBsAg and HBV DNA levels in serum correlated with the intrahepatic proportion of linear HBV DNA. Furthermore, ddPCR experiments on serum samples and experiments with nuclease indicated the contribution of encapsidated double-stranded linear DNA and replication intermediates to be limited. Conclusions: The degree of integration of intrahepatic HBV DNA in the HBeAg-negative stage may be higher than previously anticipated, and integrated DNA may explain the persistence of high HBsAg serum levels in patients with low HBV DNA levels.

Keywords: hepatitis B virus; HBsAg; integration; droplet digital PCR; liver biopsy
INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death and its major risk factor is chronic infection with hepatitis B virus (HBV) [1, 2]. While details regarding HBV-related carcinogenesis mechanisms remain unknown, genomic integration of HBV DNA may alter the function of host or viral genes or induce chromosomal instability with ensuing cancer risk [3]. Methods to estimate the extent of genomic integration of HBV DNA may help to decipher the mechanisms of carcinogenesis in addition to identifying patients at risk for HCC.

HBV has a partially circular genome called relaxed circular DNA (rcDNA), which in infected hepatocytes is transformed to a fully circular episome called covalently closed circular DNA (cccDNA) that resides in the nucleus of the cells [4, 5]. Pregenomic RNA (pgRNA) is expressed from cccDNA and converted into rcDNA in the viral capsid by the viral polymerase. pgRNA spans more than one turn of cccDNA and cannot be formed from linearized HBV DNA. Therefore, virus particles with a complete genome cannot be formed from integrated HBV DNA [6].

The integration of HBV DNA and the synthesis of viral surface antigen (HBsAg) from integrated DNA in the hepatoma cell line PLC/PRF/S were reported in 1980 [7]. Whereas viral integration has since been studied mainly with respect to its presumed relevance to the development of HCC, it has also been proposed that integrated HBV DNA contributes to the production of the HBsAg that is detected in serum of infected patients, in particular in those with low levels of viremia [6, 8-10]. It is known that PLC/PRF/S cells produce HBsAg but not viral particles containing HBV DNA, but the question whether integrated DNA is a significant source of HBsAg in infected patients has remained unresolved. A method to quantify the degree of integration may thus be useful for the clinical assessment of chronic HBV infection in addition to DNA quantification [11, 12]. HBsAg and HBV DNA
decline with strikingly different kinetics when viral replication is suppressed during immune-
mediated clearance of infection [6]. This difference might reflect the well-established fact that viral
particles and HBsAg-containing subviral particles are synthesized through different pathways from
cccDNA, and accordingly may have different production rates [5]. Alternatively, HBsAg in serum may
be expressed from integrated HBV DNA. Studies using massive parallel sequencing and Alu PCR have
identified integrations distributed widely in all human chromosomes [3, 13, 14]. Furthermore, recent
studies using inverse PCR indicate that HBV integration is frequent already during early stages of
infection, and some HBV-host junction sequences are found in a large proportion of cells suggesting
considerable clonal hepatocyte expansion [15].

A number of mechanisms linking HBV integrations to HCC development have been suggested [16,
17]. Studies have described altered expression of the oncogenes TERT and MLL4 linked to HBV
integration [6, 16]. Furthermore, HBV integration has been suggested to induce chromosomal
instability. A larger number of integrations near fragile sites such as repetitive regions, CpG islands
and telomeres have been observed in tumours than in non-tumour tissue [16]. Finally, expression of
wild type and truncated forms of HBV proteins from integrations have been reported to induce HCC.
This includes transacting wild type and truncated forms of HBx, transacting truncated forms of
HBsAg containing the PreS2 domains and overexpression of HBsAg with the PreS1 domain [17, 18].

For the present study, we developed a method that discriminates between circular HBV DNA (mainly
rcDNA) and total intrahepatic HBV DNA (ihDNA) that may be used to quantify linear HBV DNA, and
allows estimates of the amounts of HBV DNA integrated in the human genome. We found that most
of the ihDNA was linear, of which most seemed to be non-encapsidated.
MATERIALS AND METHODS

Patients and samples

Biopsies from 77 patients included in a cross-sectional study of chronic HBV infection [19] and a previous study [20] were used. The biopsies were collected 1993-1996 and follow up was done 1999-2006. The patients represented hepatitis B e antigen (HBeAg)-positive and negative stages of infection (Table 1). All participants had given written informed consent and the principles of the Declaration of Helsinki were followed. The Regional Ethical Review Board in Gothenburg approved the study.

Quantification of HBsAg and detection of HBeAg was performed by Architect assays (Abbott Laboratories) and HBV DNA quantification in serum by Cobas Amplicor HBV Monitor (Roche). The detection limit for HBV DNA was 200 copies/mL and for HBsAg 0.05 IU/mL.

Droplet digital PCR (ddPCR)

~5 mg of liver biopsies stored at -70°C was homogenized on a Magnalyser and DNA was extracted with Magnapur and the DNA II Tissue kit (Roche) [20]. For serum samples, the Total Nucleic Acid Isolation Kit was used.

The discriminating PCR is based on one primer system detecting total HBV DNA and one detecting only circular HBV DNA as illustrated in Figure 1. The analyses were carried out in duplicate reactions, each in a 20 μL reaction mix containing ddPCR supermix for probes (Bio-Rad), primers (750 nM), probe (500 nM) and 2.5 μL of extracted DNA. Droplets were formed in the AutoDG Droplet Generator (Bio-Rad). A Veriti Thermocycler (Applied Biosystems) was used for the PCR with thermal profiling beginning at 50°C followed by 10 minutes at 95°C, 40 cycles of 95°C for 15 s and 56°C for 45 s; 98°C for 10 min and ending at 4°C. After overnight incubation at 4°C, the plate was analysed in the
QX200 Droplet Reader (Bio-Rad). A negative template control (dH2O) and a positive control (plasmid or known positive sample) were included in each run. Reactions with less than 5 positive droplets were considered negative and were not included in the analysis.

The primers and probe used in ddPCR of circular HBV DNA were 1777F, GAGGCTGTAGGCATAATTTGGTC; 1924R, TTCTTTATAAGGGTCAATGTCCATG; and probe 1859-1885, ACTGTTCAGCCTCAAAGCTGTGCCTT. The total HBV DNA was amplified using 1550F, CGTCTGTGCTTCTCATCTG; 1627R, GCGTTCACGGTGGTCTCCA; and probe 1580-1604, TGCACTTCGCTTCACCTGCACGT. Betaglobin DNA was amplified using F, GCTCATGGCAAGAAAGTGCTC; R, GCAAAGGTGCCCTTGAGGT; and probe, AGTGATGGCCTGGCTACCTGGAC.

Benzonase experiments

Liver explant pieces frozen at −70°C were thawed, cut into 1 mm squares and lysed in maganalyser in 250 uL lysis buffer. The supernatant was diluted 1:5 in PBS. Fifty microliters were mixed with 2.5 µL benzonase nuclease (Sigma Aldrich) or PBS and incubated 1 h at RT followed by addition of 1.6 µL 50 mM EDTA. The samples were diluted to 200 µL with PBS before nucleic acid extraction with the MagNA pure Total Nucleic Acid Isolation Kit (Roche). DNA quantification was done with TaqMan qPCR targeting the core region (nt 2367-2428) as previously described [20], because the HBV concentration was close to the detection limit of ddPCR.
RESULTS

Absolute quantification of circular and total HBV DNA

The specificity of the ddPCR assays quantifying circular HBV DNA and total HBV DNA, respectively (Figure 1), was evaluated by analysing plasmids containing inserts of HBV DNA. Very similar copy numbers were obtained by the two assays when a plasmid containing the target regions of both ddPCRs was analysed (HBV genome linearized at nucleotide (nt) 30, Figure 2A). In contrast, a plasmid that mimics integrated DNA by containing one linear copy of the HBV genome from nt 1821 to 1820 [21] was amplified only by the total HBV DNA ddPCR (Figure 2B).

The precision of measuring the fraction of circular DNA out of total HBV DNA by combining the two ddPCR assays was first evaluated by mixing known amounts of DNA amplicons representing circular and linear HBV DNA. The two DNA amplicons were mixed at different ratios and as shown in Figure 2C ddPCR measurements of the fraction of circular DNA gave results close to the theoretical values. The precision of the assay was further evaluated by spiking a liver tissue sample containing mainly linear HBV DNA with graded amounts of HBV DNA from serum. Figure 2D shows that the assay also in this case gave results close to the theoretical values.

Patient characteristics

Table 1 shows the characteristics along with markers of HBV replication and liver damage for the 70 patients with ihDNA detected by ddPCR.
A large proportion of intrahepatic HBV DNA is linear

Analysis of liver biopsies by the circular and total HBV DNA ddPCR assays showed that a large fraction of the total HBV DNA was linear (median, 86%). The fraction of linear DNA was significantly larger in the HBeAg-negative than in the HBeAg-positive group (median 89% vs. 54%, p<0.0001, Figure 3, Supplementary Table).

The contribution of dslDNA, spliced variants and replication intermediates to the fraction of linear HBV DNA is small

Linear HBV DNA can be found in the forms of double stranded linear DNA (dslDNA) and integrated into the host genome. Furthermore, replication intermediates including the minus DNA strand but not a translocated plus strand would in our assay be detected as linear as they react with the total HBV DNA PCR assay, but not with the circular one. Similarly, HBV DNA derived from spliced RNA lacking the cis-mediated circularization signal would be detected as linear. To differentiate between the different forms of linear HBV DNA we started by analysing serum samples with the two ddPCR systems. The results showed that on average 81% of the total HBV DNA in 26 serum samples from patients with chronic HBV infection was circular (Figure 4A). This value is close to previous estimations of dslDNA [22] in serum, and suggest that the amount of replication intermediates and splice variants in serum is limited.

Next we exploited that the described forms of linear HBV DNA except integrated DNA are encapsidated. The replication intermediates, dslDNA as well as linear splice variants should therefore, in contrast to integrated HBV DNA, be protected from nuclease treatment by the viral capsid. Benzonase treatment of lysed samples from an explant HBV liver with a very low fraction of circular HBV DNA digested almost all HBV DNA as well as betaglobin DNA, used as a marker for chromosomal DNA (Figure 4B). This result suggests encapsidated replication intermediates and
dslDNA to constitute a small fraction of ihDNA. Treatment of a liver explant sample with a higher proportion of circular HBV DNA resulted in less digestion. Taken together these experiments show that the contribution of dslDNA, splice variants and replication intermediates to the fraction of non-linear HBV is small, suggesting that the contribution of integrated DNA is large.

The HBsAg to HBV DNA ratio correlates with the linear HBV DNA fraction

If HBsAg is expressed from integrated HBV DNA, patients with a large fraction of HBV DNA integrated in the genome would be expected to have a high ratio of HBsAg to HBV DNA in serum because HBsAg would be produced from both cccDNA and integrated HBV DNA, while HBV DNA in serum would be produced from cccDNA only. Indeed, there was a strong correlation between the HBsAg to HBV DNA ratio and the fraction of linear HBV DNA (p<0.0001) (Figure 5A). The correlation was observed also when only the HBeAg negative patients were included in the analysis (p=0.01).

Four HBeAg-negative patients were outliers with low fractions of linear HBV DNA. If this reflected a low degree of integrated DNA one would expect HBsAg levels to decline in parallel with HBV DNA. To test this possibility, we compared HBsAg levels in serum samples taken 5.6-11.7 years (mean 9.0 years) after liver biopsy. The HBsAg levels, available in 46 HBeAg-negative patients, were significantly (p=0.01) lower in the four patients with <40% than in the 42 patients who had larger fractions of linear DNA in the biopsy (Figure 5B).

Linear HBV DNA is cleared less efficiently than replicating virus

As expected, circular HBV ihDNA, which probably mainly is rcDNA, as cccDNA has been estimated to be <10% of rcDNA [23-25], correlated significantly with serum levels of HBV DNA (which also mainly is rcDNA) (Figure 5C). This figure also shows that the levels of linear ihDNA correlated with HBV DNA
levels in serum. If linear ihDNA mainly represents integrations, these results imply that the amount of integrated HBV DNA was lower in patients with low serum levels of HBV DNA (in whom, as mentioned earlier the fraction of linear HBV DNA out of the total ihDNA was higher).

**Estimation of the amount if integrated HBV DNA**

We estimated the fraction of integrated HBV DNA by subtracting dslDNA (presumed to be 20% of rcDNA) from the linear HBV DNA and then divided with the amount of total HBV DNA. By this calculation, integrations were found to constitute 87% of total ihDNA in HBeAg-negative as compared with 46% in HBeAg-positive patients (p<0.0001, Figure 6A). A plot of the calculated percentage of integrated HBV DNA versus the percentage of linear HBV DNA shows that the difference between the percentage of observed linear and calculated integrated HBV DNA actually was very small, in particular at high proportions of linear DNA (Figure 6B). By relating the calculated integration copy numbers to the copy numbers of the betaglobin gene, which is present in two copies per cell, and assuming that hepatocytes constitute 70% of the cells in the biopsies, the median number of integrations per hepatocyte was estimated to be 1.7 in HBeAg-positive and 0.2 in HBeAg-negative patients.

**DISCUSSION**

Integration of HBV DNA has been proposed as a significant oncogenic mechanism in HCC and a source of HBsAg in serum of HBV carriers with minimal viral replication. This study presents a novel analytical strategy for quantifying ihDNA, which distinguishes circular HBV DNA from HBV DNA that is linearized around nt 1820, where integrations typically start or end. When used with the quantitative accuracy of ddPCR this strategy provides an opportunity to estimate the extent of HBV integration.
The main finding was that linear forms, which include integrations, constituted a large proportion of ihDNA, in particular in HBeAg-negative patients. This supports that integrated HBV DNA is the main form of ihDNA in late stage of chronic infection. In addition to integrated HBV DNA, the assay detects dsI DNA, replication intermediates (minus DNA strand) and splice variants as linear HBV DNA. These other forms of linear HBV DNA are possible byproducts of the rcDNA synthesis and are therefore expected to be dependent on the rcDNA concentration. Interestingly the ratio between linear DNA and rcDNA was around 10 times higher in the HBeAg-negative stage, which is characterized by low rcDNA synthesis, than during the HBeAg-positive phase, which is characterized by high rcDNA synthesis.

To directly address the composition of the linear HBV DNA, we exploited that integrated HBV DNA is the only linear form that is not encapsidated and therefore sensitive to nuclease treatment. Experiments with benzonase suggested encapsidated forms of HBV DNA (dsI DNA, ssDNA and splice variants) to make small contributions to the linear DNA fraction in samples with a large fraction of linear HBV DNA, supporting integrated DNA to be the dominating species. In addition, our analysis of serum samples with the two ddPCR systems showed that around 20% of the HBV DNA was linear, which is in line with previous quantifications of dsI-DNA [22].

The correlation between the proportion of linear ihDNA and the ratio between HBsAg and HBV DNA in serum suggests that integrated HBV DNA may contribute to the production of HBsAg, thus explaining why high serum levels of HBsAg are frequently found in HBeAg-negative patients with low HBV DNA levels in serum. This possibility has been discussed for decades [26] but has, to our knowledge, not been proven. The relevance of integrations for HBsAg production was further
supported by the observation that serum levels of HBsAg 9 years after the biopsy were significantly lower in four HBeAg-negative patients with lower (<40%) than in those with a higher fraction (>40%) of linear HBV DNA.

HBsAg production from integrated HBV DNA has been supported by recent observations. One study showed a slower reduction of serum HBsAg levels during antiviral therapy in the 15% of the patients in whom an HBV S gene integration was detected by Alu PCR [9]. Another study reported that, in selected patients, the molecular weight of HBsAg in subviral particles did not reflect observed deletions in the HBV DNA in serum [10]. Finally, a transcriptome analysis of chimpanzee liver mRNA demonstrated that in HBeAg-negative infections most HBV transcripts ended near nt 1820, as the end of the suggested template for integration dsDNA, and also identified HBV-host fusion sequences [8]. Interestingly, the finding that inactive HBsAg carriers have lower proportions of the long HBsAg variants (L and M) than actively HBV DNA replicating patients, suggests that many integrations might be truncated in the 5' end [27, 28].

Our calculations suggested that the fraction of integrated HBV DNA out of the total iodNA was 46% in HBeAg-positive and 87% in HBeAg-negative patients and that the number of integrations per hepatocyte was 1.7 in HBeAg-positive and 0.2 in HBeAg-negative patients. The HBV integration frequency has previously been estimated by end-point titration of circularized fragments generated by inverse-PCR [15]. As computer simulations indicated this method to detect 10% of integrations [29] the study suggested 10% of hepatocytes to have integrations, which is quite close to our value (20%) for HBeAg-negative patients. Cross-sectional studies have shown that the ratio between HBsAg and HBV DNA in serum is a hundred-fold higher in HBeAg-negative than positive patients [20, 30]. Notably, to be responsible for this difference, integrations, if the transcription rate is the same
as from cccDNA, need to be present in amounts 2 \log_{10} units lower than cccDNA in the HBeAg-positive stage (1 integration/10 cells) [23], which is close to the number that we report (1/5 cells).

Although the fraction of integrated HBV DNA was lower in HBeAg-positive patients, the average number of integrations per hepatocyte was 10 times higher in this group than in HBeAg-negative patients, reflecting that the total ihDNA was on average 1.5 \log_{10} units higher in HBeAg-positive patients. This observation suggests that many integrations occur early during the infection, when the concentration of virus is the highest. It also suggests that the mechanisms that reduce viral replication also eradicate hepatocytes with HBV integrations, but that the latter process is slower. Hepatocytes with integrations could be eradicated because cytotoxic T cells recognize epitopes derived from all viral proteins expressed from cccDNA, or from HBsAg or X protein expressed from integrations. However, HBsAg expressed from integrations might also have an opposite effect by inducting immunological hyporeactivity [31].

Our analytical strategy has limitations in that we assume that the circular forms of HBV DNA essentially are only rcDNA. This is based on previous reports that cccDNA constitute <10% of rcDNA [23-25] and has no impact on the assessment of linear or integrated HBV DNA, which was based of subtraction of circular HBV DNA from total ihDNA. To account for dsIDNA, which according to previous studies [5, 22] and our measurements constitute 20% of rcDNA, we subtracted 1.2 times circular DNA from total HBV DNA to calculate integrated HBV DNA. This might, if dsIDNA were formed in a larger amount result in an overestimation of integrations, but as dsIDNA is expected to be a fraction of rcDNA, this overestimation would be significant only if circular forms of HBV DNA were frequent. Replication intermediates such as single stranded HBV DNA (ssDNA) would be interpreted as linear HBV DNA by our assay. The importance of this limitation is uncertain since
ssDNA in liver biopsies has not been well characterized [32-34]. This DNA form was suggested to exist based on Southern blot experiments showing smears migrating faster than full length HBV DNA [32]. Based on the assumption that replication intermediates are encapsidated and the findings in our benzonase experiments, we assumed that the level of ssDNA is negligible. If not correct, this would result in an overestimation of the amount of integrated DNA. Similarly, splice variants lacking the cis mediated circularization signal would also be interpreted as linear in our assay. However, since they are rare (<5%) in patients without HCC, their contribution to the linear DNA should be small [35, 36].

In summary, we introduce a method to measure circular and linear HBV DNA in liver biopsies. Our findings support that a large fraction of the ihDNA is integrated, which may explain persistence of high levels of HBsAg in serum in patients with low HBV DNA levels. Given the probable association between viral integration and development of HCC, the finding of a high degree of viral integration among HBeAg-positive patients could argue for initiating antiviral treatment earlier during chronic infection. The proposed method might be helpful in studies aiming at further understanding the mechanisms of oncogenesis in chronic HBV infection.
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FIGURE LEGENDS

Figure 1. Principle of discriminating PCR. The two PCR assays, one using primers 1550F and 1627R (total HBV DNA), the other using primers 1776F and 1924R (circular HBV DNA), differ in their ability to amplify the different forms of HBV DNA. Both assays amplify cccDNA and rcDNA, but the combination of 1776F and 1924R does not amplify double stranded linear DNA (dslDNA) or integrated HBV DNA, formed from dslDNA, because on these targets the primers are directed away from each other. Flanking human DNA is shown as grey extensions in the figure that illustrates integrated HBV DNA. The HBsAg ORF is shown as a green arrow. The position of nt 30 is indicated in red to show that a linearization at this position, as in the first control plasmid, does not affect the total or circular PCR systems.

Figure 2. Verification of discriminative digital PCR. A) Quantification of serial dilutions of a plasmid containing the whole HBV genome linearized at position 30, showing almost identical copy numbers by the two ddPCR assays nt 1550-1627 and 1776-1924, detecting total and circular HBV DNA, respectively. B) A plasmid linearized at position 1825 (i.e. mimicking HBV DNA integrated in chromosomal DNA) was as expected not amplified by the ddPCR detecting circular HBV DNA (nt 1776-1924), but only by the ddPCR targeting total HBV DNA (nt 1550-1627.) C) A PCR product representing linear DNA made with the total HBV DNA primers (nt 1550-1627) and a PCR product representing circular HBV DNA made with the forward primer of the total HBV system and the reverse primer of the circular system (nt 1550-1924) were mixed in the ratios indicated. The plot shows the ratio circular:total HBV DNA for the dilution series as calculated (theoretical) and measured by the two ddPCR systems (measured). D) A liver biopsy containing mainly linear HBV DNA (totally 630 copies/reaction, with 14% circular DNA as measured by ddPCR) was spiked with a
dilution series of HBV DNA from a serum sample (4600, 1500, 510 and 170 copies/reaction of total HBV DNA of which 80% was circular DNA) and assayed with the ddPCRs. The plot shows the ratio circular:total HBV DNA for the dilution series as calculated from the composition of the biopsy and serum sample (theoretical) and measured by ddPCR (measured).

Figure 3. The proportions of linear HBV DNA out of the total HBV DNA in liver biopsies were larger in 56 HBeAg-negative than in 14 HBeAg-positive patients (p<0.0001, Mann-Whitney U test). The proportion of linear HBV DNA was determined using the ddPCR assays detecting total HBV DNA (nt 1550-1627) and circular HBV DNA (nt 1776-1924).

Figure 4. Estimation of the fraction of dsDNA in serum and unencapsidated HBV DNA in explant tissue. A) Serum samples from 26 patients chronically infected with HBV were analysed with the two ddPCR assays, and the % of circular HBV DNA was calculated. The line represents the mean value. B) Lysed hepatocytes from liver tissue from two patients (Tissue 1 from a patient on antiviral therapy, with undetected HBV DNA in serum, and with ≈ 1 HBV DNA copy per 100 hepatocytes; Tissue 2 from a patient with HBV DNA 5.5 log IU/mL serum, and with ≈ 1 HBV DNA copy per hepatocyte), and a mixture of liver tissue and serum HBV DNA in excess (from an unrelated patient with very high viral load), were analysed. The samples were or were not pre-treated with benzonase (which digests all types of nucleic acid) prior to nucleic acid extraction and quantification of viral (core gene target) and human (betaglobin gene) DNA. The plot shows the fraction of DNA digested by the enzyme (lines show mean).
Figure 5. A. The ratio between HBsAg and HBV DNA in serum correlated significantly with the proportion of linear intrahepatic HBV DNA (HBeAg-positive, filled triangles; HBeAg-negative, open triangles). The regression line, as well as the $R^2$ and Pearson’s correlation coefficient ($p$) values is based on values for all patients. B. The HBsAg levels in serum taken 5.6-11 years after the liver biopsy were lower in 4 HBeAg-negative patients with linear HBV DNA fractions below 40% than in the 42 other HBeAg-negative patients ($p=0.01$, Mann-Whitney U test). Lines represent median. C. Linear regression and correlation between HBV DNA in serum and intrahepatic HBV DNA in the form of circular (black filled circles) and linear HBV DNA (open triangles) in liver biopsies. $p$, Pearson’s correlation coefficient.

Figure 6. Calculation of the proportion of integrated HBV DNA. The proportion of integrated DNA was estimated using a formula that subtracts $0.2 \times$ circular HBV DNA from the linear HBV DNA. The lines represent median values. B shows the calculated percentage of integrated HBV DNA as a function of the percentage of linear of HBV DNA.
Table 1. Characteristics of 70 patients with HBV DNA detected by ddPCR

| Characteristic                                      | HBeAg+ | HBeAg- |
|-----------------------------------------------------|--------|--------|
|                                                     | n=14   | n=56   |
| Age (mean, range)                                   | 33 (16-60) | 34 (18-50) |
| Gender (female/male)                                | 5/9    | 23/33  |
| Geographic origin (A/EA/ME/NE/SE)*                  | 2/3/4/4/1 | 3/9/27/8/9/ |
| HBV DNA in serum (mean log10 copies/mL)             | 8.21   | 4.41   |
| HBsAg (mean log10 IU/mL)                            | 4.38   | 3.52   |
| Genotype (A/B/C/D)                                  | 3/2/2/7 | 11/6/3/36 |
| ALT/upper limit of normal (mean)                    | 2.26   | 1.19   |
| ALT upper limit of normal ≥2                        | 5      | 4      |

*A, Africa; EA, East Asia; ME, Middle East; NE, North Europe; SE, South Europe*
Figure 2

A

- Circular HBV DNA
- Total HBV DNA

$R^2 = 0.9995$

$R^2 = 0.9994$

Copies per reaction

Relative concentration of plasmid

B

Copies per reaction

Circular HBV DNA
Total HBV DNA

C

- Measured
- Theoretical

Relative amounts of DNA linear/circular

D

- Measured
- Theoretical

Relative amounts of DNA biopsy/serum
Figure 3

% of total intrahepatic HBV DNA

HBeAg+    HBeAg-
Linear

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Figure 5

A) The graph shows the relationship between the ratio of HBV DNA (log10 IU per ml) to HbsAg (log10 copies per ml) and the proportion of linear HBV DNA. The equation is given as $R^2 = 0.31 \quad p = 0.0001$.

B) The graph illustrates the relationship between the follow-up HbsAg (log10 IU/mL) and the proportion of linear HBV DNA. The data points are divided into two groups: <40% and >40%.

C) The graph demonstrates the relationship between linear and circular HBV DNA (log copies/10^12) and the HBV DNA in serum (log copies/mL). The equations for linear and circular DNA are given as $y = 0.30x + 0.85 \quad R^2 = 0.54 \quad p = 0.0001$ for linear DNA, and $y = 0.45x - 0.64 \quad R^2 = 0.76 \quad p = 0.0001$ for circular DNA.
