

# Next Generation Digital PCR Measurement of Hepatitis B Virus Copy Number in Formalin-Fixed Paraffin-Embedded Hepatocellular Carcinoma Tissue

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**BACKGROUND:** Hepatocellular carcinoma (HCC) is strongly associated with hepatitis B virus (HBV) infection. False-negative results are common in routine serological tests and quantitative real-time PCR because of HBV surface antigen (HBsAg) variation and low HBV copy number. Droplet digital PCR (ddPCR), a next generation digital PCR, is a novel, sensitive, and specific platform that can be used to improve HBV detection.

**METHODS:** A total of 131 HCC cases with different tumor stages and clinical features were initially classified with a serological test as HBsAg positive ( $n = 107$ ) or negative ( $n = 24$ ) for HBV infection. Next, DNA templates were prepared from the corresponding formalin-fixed paraffin-embedded (FFPE) tissues to determine HBV copy number by ddPCR.

**RESULTS:** HBV copy numbers, successfully determined for all clinical FFPE tissues ( $n = 131$ ), ranged from 1.1 to 175.5 copies/ $\mu\text{L}$  according to ddPCR. The copy numbers of HBV were positively correlated with tumor-nodes-metastasis ( $P = 0.008$ ) and Barcelona-Clinic Liver Cancer ( $P = 0.045$ ) classification. Moreover, serum cholinesterase correlated with hepatitis B viral load ( $P = 0.006$ ).

**CONCLUSIONS:** HBV infection is a key factor that influences tumorigenesis in HCC by regulating tumor occurrence and development. ddPCR improves the analytical sensitivity and specificity of measurements in nucleic acids at a single-molecule level and is suitable for HBV detection.

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Hepatocellular carcinoma (HCC)<sup>6</sup> is one of most common neoplasms, with >700 000 cases diagnosed in 2008 (1). In the US, approximately 6000 new HCC cases are diagnosed each year that either are intrinsically resistant to chemotherapy or initially respond to it but later develop resistance (2). The mortality of HCC is extremely high, with only a 5%–9% overall 5-year survival rate from the time of clinical diagnosis (3). The incidence of HCC is related to the hepatitis B viral load and the duration of infection, indicating that hepatitis B virus (HBV) has a cumulative effect on tumorigenesis (3, 4). More than 80% of HCC patients in eastern Asia and sub-Saharan Africa also have HBV infection (3, 5, 6). HBV is a small, enveloped DNA virus that consists of 3.2-kb partially double-stranded relaxed circular DNA (rcDNA). The rcDNA can be converted into a stable covalently closed circular DNA (cccDNA) in the nucleus, after which it serves as the original template for viral replication and plays an important role in HBV persistence in the nucleus of infected hepatocytes (7), which may explain HBV reactivation and why HBV cannot be completely eliminated by antiviral agents (7, 8). Inactive HBV infection also has a substantial risk of HCC due to HBV integration and DNA damage (9).

Currently, the diagnosis of HBV infection is routinely performed with serological tests and quantitative real-time PCR (qPCR) (10, 11). However, false-negative results are common with these methods because of HBV surface antigen (HBsAg) variation and low HBV copy number. In particular, the precision limitations of qPCR have prevented distinguishing between small differences in copy number among samples (12), especially in cases of low copy number.

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<sup>6</sup> Nonstandard abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; rcDNA, relaxed circular DNA; cccDNA, covalently closed circular DNA; qPCR, quantitative real-time PCR; HBsAg, HBV surface antigen; FFPE, formalin-fixed paraffin-embedded; ddPCR, droplet digital PCR; HBsAb, HBV surface antibody; AFP,  $\alpha$ -fetoprotein; TNM, tumor-nodes-metastasis; BCLC, Barcelona Clinic liver cancer; 5-mC, 5-methylcytosine; 5-hmC, 5-hydroxymethylcytosine.

It is difficult to accurately justify the results of qPCR when the quantification cycle (C<sub>q</sub>) value of an unknown sample is near the cutoff. This phenomenon is frequent in the evaluation of HBV from formalin-fixed paraffin-embedded (FFPE) tissues. In clinical practice, patients with decompensated cirrhosis and detectable HBV DNA require urgent antiviral treatment (8). Thus, there is a need to develop more analytically sensitive and accurate methods to determine HBV infection.

Droplet digital PCR (ddPCR) is a new method of digital PCR that enables the absolute quantification of nucleic acid without the use of calibration curves (13). This method relies on limiting dilutions of the PCR volume and Poisson statistics (9, 14). After the dilutions, the PCR mix is distributed into fractions, and each reaction is independently interrogated for the copy number of the target nucleic acids at a single-molecule sensitivity (15). The absolute number of target nucleic acid in the original sample can be calculated with Poisson statistics from the ratio of positive to total partitions (14, 16–19). Evidence has shown that ddPCR is more analytically sensitive than qPCR (18, 20). Because of its single-molecule sensitivity, ddPCR is particularly applicable for detecting the copy number of a viral load (21, 22). Importantly, ddPCR also has the potential to improve HBV measurements because of its ability to quantify nucleic acid targets with high precision and accuracy. Taken together, these advantages show that ddPCR has better analytical sensitivity, precision, accuracy, and day-to-day reproducibility compared with qPCR, indicating that the results from ddPCR are more reliable and applicable than those from qPCR (17). In this study, we used a ddPCR system to measure the HBV copy number in FFPE hepatocellular carcinoma samples, and also investigated the association of HBV copy number with tumor stage and clinical features.

## Materials and Methods

### PATIENTS AND DNA SAMPLES

A total of 132 HCC patients were enrolled in this study between June 2005 and January 2012 at the Zhongnan Hospital of Wuhan University. Informed consent was obtained from each participant, and the ethics committee of Zhongnan Hospital, Wuhan University, China, approved the study. DNA templates were extracted with DEXPAT Easy kit (Takara) from FFPE tumor tissues of HCC patients, as confirmed by pathology. The DNA was then purified with the AxyPrep DNA Gel Extraction Kit (Axygen) according to the manufacturer's instructions. A total of 27 DNA samples from healthy individuals who had nonpathologic liver function and no history of HBV infection [HBsAg, HBV surface antibody (HBsAb), HBeAg, HBeAb, and HBcAb were negative by serological tests, and

HBV DNA was undetectable by qPCR] served as negative controls.

### ddPCR

We quantified HBV copy number with the QX100™ Droplet Digital™ PCR system (Bio-Rad). The 20-μL ddPCR mixture consisted of 10 μL 2× ddPCR supermix for probes (Bio-Rad); 900 nmol/L HBV sense (5'-CTCTCTTTACGCGGTCTC-3') and HBV antisense (5'-GTCGTTGACATTGCTGAG-3') primers, which produced a 161-bp amplicon; 250 nmol/L HBV probe (5'-CCGTCTGTGCCTTCTCATCTGC-3'); and 4 μL DNA sample. The mixture was placed into the DG8 cartridge, 70 μL of droplet generation oil was added, and droplets were formed in the droplet generator (Bio-Rad). After processing, the droplets were transferred to a 96-well PCR plate (Eppendorf). We carried out PCR amplification on a C1000 thermal cycler (Bio-Rad) with a thermal profile beginning at 95 °C for 10 min, followed by 45 cycles of 94 °C for 30 s and 57 °C for 60 s, 1 cycle of 98 °C for 10 min, and ending at 4 °C. After amplification, the plate was loaded on the droplet reader (Bio-Rad). We included no-template controls for detecting PCR contamination. We used pBlue-HBV plasmid containing 1.3-fold HBV genome (from Dr. Yin Zhu's laboratory, State Key Laboratory of Virology, College of Life Sciences, Wuhan University, China) as a reference standard. ddPCR data were analyzed with QuantaSoft analysis software (Bio-Rad). The quantification measurements of the target molecule were presented as the copy numbers per microliter of DNA sample.

### QUANTIFICATION OF HBV cccDNA

We quantified HBV cccDNA in FFPE tumor tissues of HCC patients as described previously (23).

### SEROLOGICAL TESTS AND CLINICAL FEATURES

We assessed serological tests of HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, and anti-HCV using Architect chemiluminescent enzyme immunoassays (Abbott Architect i system). Clinical preoperative biochemical parameters and tumor biomarkers were measured with an automated chemistry analyzer (Abbott-Aerosep, Abbott Diagnostics) with commercial kits, including serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, globulin, γ-glutamyltransferase, alkaline phosphatase, 5-nucleotidase, total biliary acid, cholinesterase, prealbumin, glucose, blood urea nitrogen, creatinine, uric acid, retinol-binding protein, cystatin C, carcinoembryonic antigen, and α-fetoprotein (AFP).

### STATISTICAL ANALYSES

All data were analyzed with SPSS version 19.0. We used generalized linear models to analyze the association of

HBV DNA and cccDNA copy numbers with tumor stages and clinical features after adjusting for sex, age, smoking, and alcohol consumption. We used the  $\kappa$  statistic to investigate the concordance between the HBV results from ddPCR and HBsAg serological testing. The mean (SE) was used for normally distributed data, and the median with interquartile range was used for skewed data. All statistical tests were 2-sided, and the level of statistical significance was set at  $P < 0.05$ .

## Results

### PATIENT CHARACTERISTICS

Among the 132 patients enrolled in this study, the mean age was 49.4 (12.3) years, and the male:female ratio was 108:24. The serological tests for HBV and HCV indicated that the majority of HCC patients (81.1%, 107/132) were HBsAg positive. The etiologies in HBsAg-negative HCC were non-alcoholic fatty liver disease (28.0%, 7/25), alcohol intake (12.0%, 3/25), diabetes mellitus (8.0%, 2/25), HCV infection (4.0%, 1/25), and schistosome infection (4.0%, 1/25). The remaining patients (44.0%, 11/25) had a cryptogenic etiology. To exclude the interference of HCV, we removed 1 patient who was seropositive for anti-HCV. Of the 131 HCC patients included in the analysis, 87 had stage I disease, 13 had stage II disease, 18 had stage III disease, and 13 had stage IV disease on the basis of the tumor-nodes-metastasis (TNM) staging system. According to the Barcelona Clinic liver cancer (BCLC) staging system, 1 patient had stage 0 disease, 36 had stage A disease, 76 had stage B disease, and 18 had stage C disease. Patient demographic and clinicopathological information is presented in Table 1.

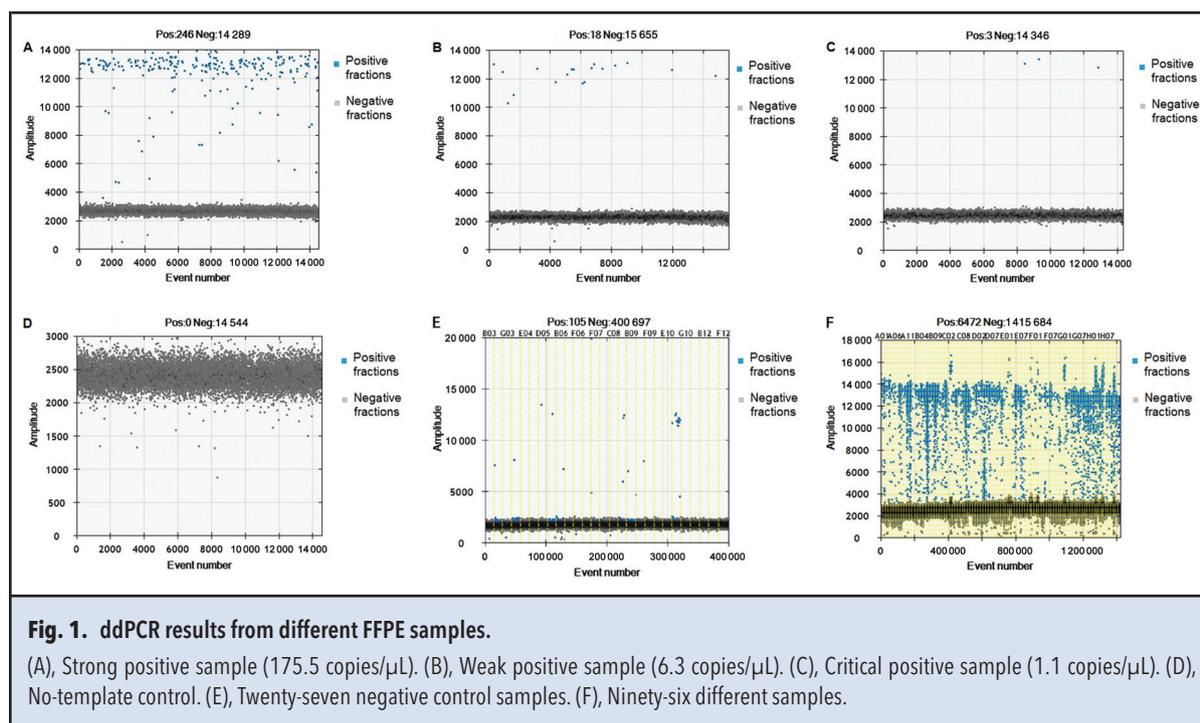
### ddPCR ANALYSIS OF HBV DNA

We initially analyzed the DNA samples without purification, but the results were not acceptable. There were many impurities in the DNA samples, which led to large variations in the data. The data variation was small and the reproducibility was high when we used purified DNA samples (Fig. 1). With QuantaSoft analysis, we found that all of the DNA samples ( $n = 131$ ) from HCC patients had hepatitis B viral load, regardless of a HBsAg-positive ( $n = 107$ ) or -negative ( $n = 24$ ) serological test result, and that the HBV copy numbers ranged from 1.1 (SD 0.7) to 175.5 (SD 8.2) copies/ $\mu\text{L}$ . To investigate if the false-positive result could be completely excluded in the detection of FFPE samples by ddPCR, hepatitis B viral load was detected in the negative controls ( $n = 27$ ), and the mean copy number of HBV DNA was 0.2 (SD 0.1) copies/ $\mu\text{L}$  at 99% confidence level. We validated that the HBV DNA copy numbers in FFPE tissues of HCC patients were much higher than those in the negative controls. To verify the accuracy of our ddPCR data, the copy number of pBlue-HBV plasmid as a reference

**Table 1. Characteristics of HCC patients.**

Baseline variable	HCC patients, n (%)
Sex	
Male	107 (81.7)
Female	24 (18.3)
Smoking	
Yes	46 (35.1)
No	85 (64.9)
Drinking	
Yes	35 (26.7)
No	96 (73.3)
TNM staging	
I	87 (66.4)
II	13 (9.9)
III	18 (13.8)
IV	13 (9.9)
BCLC staging	
0	1 (0.8)
A	36 (27.5)
B	76 (58.0)
C	18 (13.7)
Child-Pugh classification	
A	116 (88.5)
B	15 (11.5)
Tumor size (cm)	
<5.0	37 (28.2)
$\geq$ 5.0	94 (71.8)
HBsAg	
Positive	107 (81.7)
Negative	24 (18.3)
HBsAb	
Positive	9 (6.9)
Negative	122 (93.1)
HBcAb	
Positive	85 (64.9)
Negative	46 (35.1)
HBeAg	
Positive	37 (28.2)
Negative	94 (71.8)
HBeAb	
Positive	80 (61.1)
Negative	51 (38.9)

standard was analyzed by ddPCR and showed a high degree of linearity and correlation ( $R^2 = 0.9991$ ) (see Supplemental Fig. 1, which accompanies the online version of this article at <http://www.clinchem.org/content/>



vol61/issue1). We also further investigated the concordance between the HBV results from the ddPCR and HBsAg serological tests with the  $\kappa$  statistic. The  $\kappa$  statistic tests the agreement between 2 methods;  $\kappa < 0.80$  indicates poor agreement. Our data showed that there were significant differences between the methods ( $P < 0.001$ ) and that ddPCR had a higher positive incidence. The incidence of false-negative serological test results was 18.3%, which was not satisfactory for clinical diagnosis. HBV infection was present even for patients who were seronegative for HBsAg.

#### COPY NUMBERS OF HBV CORRELATED WITH TUMOR STAGE AND SERUM CHOLINESTERASE

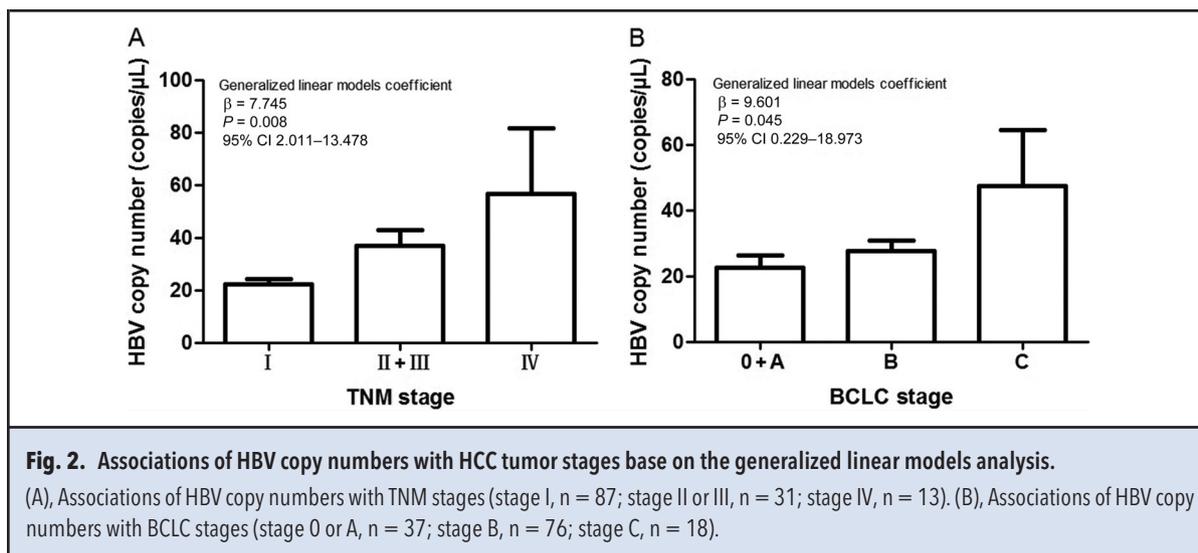
Next, we used generalized linear models to analyze the associations of hepatitis B viral load with tumor stage and clinical features after adjusting for sex, age, smoking, and alcohol consumption. Notably, we found that the hepatitis B viral load was correlated with the TNM stage ( $n = 131$ ,  $\beta = 7.745$ ; 95% CI 2.011–13.478;  $P = 0.008$ ) (Fig. 2A). Moreover, the copy numbers of HBV were associated with BCLC stage ( $n = 131$ ,  $\beta = 9.601$ ; 95% CI 0.229–18.973;  $P = 0.045$ ) (Fig. 2B). In addition to tumor stage, the viral load of HBV was positively correlated with serum cholinesterase ( $n = 130$ ,  $\beta = 0.004$ ; 95% CI 0.001–0.006;  $P = 0.006$ ). No significant association was found between the HBV copy number and the other clinical features examined in our study.

#### COPY NUMBERS OF HBV cccDNA CORRELATED WITH CHILD-PUGH SCORE AND SERUM AFP CONCENTRATIONS

Because the quantification of HBV DNA included single- and double-stranded DNA, it was important to explore the relationship between HBV cccDNA in liver tissues and disease progression. We successfully detected HBV cccDNA copy numbers per hepatocyte in 39 of 131 samples (see online Supplemental Table 1). We applied generalized linear models to analyze the association of cccDNA copy numbers with tumor stage and clinical features after adjusting for sex, age, smoking, and alcohol consumption. As a result, cccDNA copy numbers were correlated with the Child-Pugh score ( $n = 39$ ,  $\beta = 7.619$ ; 95% CI 1.719–13.518;  $P = 0.011$ ) (Fig. 3A) and serum AFP ( $n = 35$ ,  $\beta = 7.818 \times 10^{-5}$ ; 95% CI  $4.038 \times 10^{-5}$ – $1.160 \times 10^{-4}$ ;  $P = 5.049 \times 10^{-5}$ ) (Fig. 3B). However, no significant association was found among the HBV cccDNA copy numbers, tumor classifications, and other clinical features examined in our study.

#### Discussion

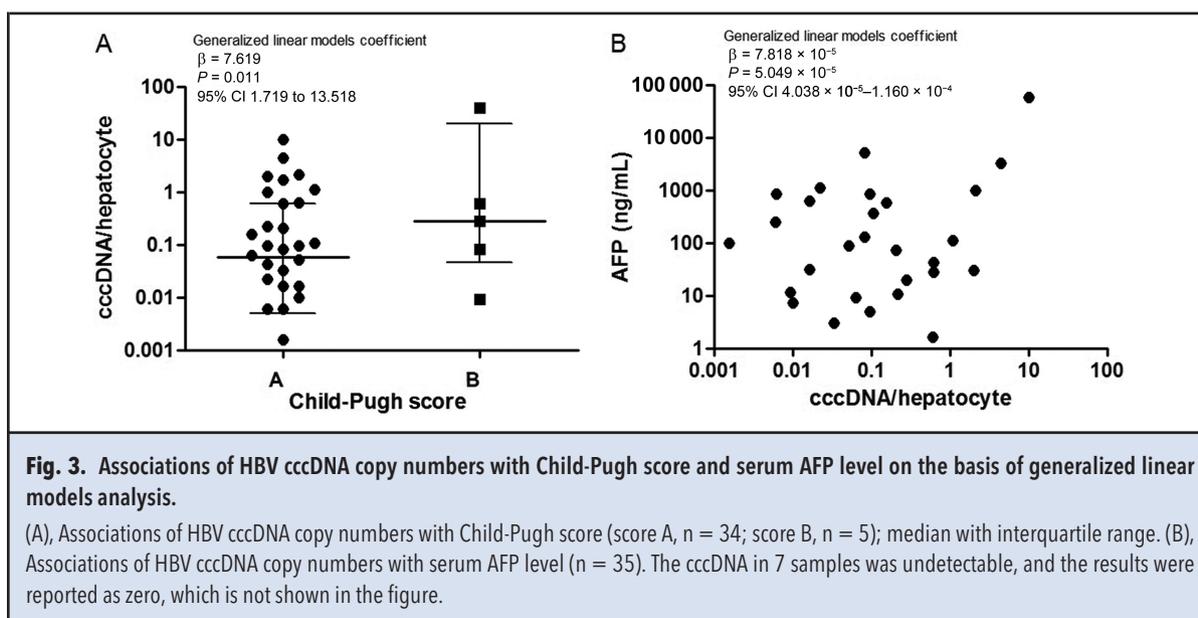
The incidence of and mortality associated with hepatocellular carcinoma are increasing worldwide, and accurate, non-invasive biomarkers for the early diagnosis of HCC are urgently needed to reduce HCC-related morbidity and mortality (24). Recently, we developed an online trapping/capillary hydrophilic-interaction liquid chromatography/



insource fragmentation/tandem mass spectrometry system for HCC detection by quantifying 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) in genomic DNA from HCC tumor samples. However, the limits of detection for 5-mC and 5-hmC were 0.06 and 0.19 fmol, and the imprecision and recovery of the method were poor, with relative SDs and relative errors of 14.9% and 15.8%, respectively (25). Thus, we developed a novel assay that is more analytically sensitive for identifying patients with a low hepatitis B viral load and can be applied for the early detection and prognosis of HCC.

Our results demonstrated that ddPCR can be used to determine the HBV copy number in clinical FFPE samples

and suggested that the copy numbers of HBV DNA in liver tissue correlate with TNM and BCLC stages of HCC. Our data showed a direct positive correlation between the hepatitis B viral load and cccDNA in liver tissue and the development of HCC, which indicated that cccDNA secures HBV persistence and reactivation in hepatocytes and that HBV replication is associated with tumor development. Because HBV integrates into the host genome and the copy numbers of HBV increase as the tumor develops, monitoring HBV DNA and cccDNA has the advantage of indicating the development of malignancy. HBV infection has been associated with >80% of HCC patients (26); the ideal end point for treatment of chronic HBV infection is



cccDNA elimination (7) and HBsAg loss (8). Our results further confirmed the oncogenicity of HBV, which supports the viewpoints that the expressions of HBV proteins themselves have oncogenic potency and that the integration of HBV DNA into the host genome promotes carcinogenic mechanisms in the host genome (26–29).

With ddPCR, we discovered that all of the HCC patients in our study had a hepatitis B viral load, even in cases that were seronegative for HBsAg. This result demonstrated that the serological tests for HBV are not analytically sensitive enough to diagnose HBV infection because variant HBV antigens cannot be recognized by the specific antibody. We also found that all of the DNA samples had detectable HBV copy numbers, which indicated that each patient in our study was infected with HBV. Our results demonstrated the oncogenicity of HBV.

Next, we found that as the copy number of HBV increased, the serum cholinesterase activity increased as well. Cholinesterase is a protein that is primarily synthesized by the liver, and the serum cholinesterase activity indicates the liver function reserve (30). The serum cholinesterase activity is related to the synthetic activity of the hepatocytes and some hepatic diseases, such as chronic hepatitis and cirrhosis, resulting in changes in the enzyme activity (31). Cholinesterase activity is closely related to the damage of hepatocytes and the HBV DNA copy number, resulting in more damage to hepatocytes. This is not confirmatory of our results, possibly because of the abnormal proliferation of malignant cells, which leads to a high amount of synthesized cholinesterase. This abnormal phenomenon should be studied further.

In this study, we developed a ddPCR assay for the measurement of HBV copy number in FFPE hepatocellular carcinoma tissue. With this method, we successfully detected the hepatitis B viral load in FFPE DNA samples from 131 clinical cases of HCC. Our results indicated that hepatitis B virus infection is a fundamental factor in hepatocellular carcinogenesis because it influences tumor occurrence and development. We also demonstrated that ddPCR can improve the analytical sensitivity and specificity of measurements in nucleic acids at the single-molecule level and is suitable for HBV detection.

Thus, next generation digital PCR, as a high-sensitivity measure of detecting low-copy-number HBV viral load, not only helps HBsAg-negative patients with liver disease by obtaining an earlier diagnosis, but also provides an important theoretical basis for HCC chemotherapy and/or immunosuppression. Moreover, HBV DNA reduction to undetectable concentrations by ddPCR should ideally be achieved to avoid drug resistance and prevent recurrent hepatitis after liver transplantation. Overall, the detection of HBV copy number is essential for clinical diagnosis, decision to treat and subsequent monitoring, assessment endpoints of therapy, and evaluating antiviral efficacy for chronic HBV infection patients (8). The results of the current study suggest that HBV DNA monitoring by ddPCR will be critical to discover the failure of HBV treatment and liver transplantation as early as possible, and our ddPCR technique for analysis of HBV DNA can be used as part of routine diagnostic procedures to establish early detection of hepatocellular carcinoma.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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