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Evaluation of the in situ assay for HBV DNA

An observational real-world study in chronic hepatitis B

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Abstract

The visualization of intrahepatic hepatitis B virus (HBV) DNA by in situ hybridization (ISH) has uncovered some interesting aspects of HBV life cycle at the single-cell level. In the current study, we intend to evaluate the reliability and robustness of this assay in the real-world clinical scenario and its relationship with currently available clinical biomarkers in chronic hepatitis B (CHB) patients.

In this cross-sectional study, 94 CHB patients and 10 patients with non-HBV related liver diseases were enrolled. Liver biopsies and routine histopathology analysis were performed. Intrahepatic HBV DNA and viral antigens (HBsAg and HBcAg) were detected by ISH and immunohistochemistry (IHC), respectively. The basic biochemical and virological parameters such as alanine transaminase, serum HBV DNA, and serum HBsAg were measured.

The HBV DNA-ISH assay showed 55.8% (53/94 cases) positive rate in CHB patients, no false positive was found in non-HBV related hepatitis. The IHC of HBsAg and HBcAg showed a positive rate of 94.7% (89/94 cases) and 19.5% (17/87 cases), respectively. Quantification of HBV DNA-ISH signal showed a significant correlation with serum HBV DNA ($r_s = 0.6223$, $P < .0001$). In addition, the staining pattern of HBV DNA in situ in the context of collagen deposition informed the histopathological progression of chronic liver disease.

The application of this ISH assay in evaluating intrahepatic viral replication in real-world CHB patients showed favorable performance. It can be a complementation to conventional liver histopathology examination and IHC detection of viral antigens. This methodology provides an intuitive assessment of virological and pathological state of CHB patients, and further supports clinical diagnosis and management.

Abbreviations: ALT = alanine transaminase, AST = aspartate transaminase, CHB = chronic hepatitis B, DNA = deoxyribonucleic acid, HBcAg = hepatitis B core antigen, HBeAg = hepatitis B e antigen, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, IHC = immunohistochemistry, ISH = in situ hybridization, LHBsAg = large hepatitis B surface antigen, NBT/BCTP = nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Keywords: chronic hepatitis B, DNA, HBV, in situ hybridization, intrahepatic

1. Introduction

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, is responsible for causing acute and chronic hepatitis in population. Chronic HBV infection (CHB) is a serious global public health problem. Although the incidence of HBV infection is decreasing thanks to vaccination, there are still over 250 million people chronically infected with HBV worldwide, among whom, only 10% have been diagnosed.[1,2] CHB could lead to

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This study was conducted in compliance with the ethical guidelines of the 2013 Declaration of Helsinki and ethical approval was obtained from the independent ethics committee of the 960th Hospital of Chinese PLA Joint Logistics Support Force.

Informed consent was obtained from all patients prior to their inclusion in the study.

The authors have no conflicts of interest to disclose.

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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fibrosis and cirrhosis, and it has further oncogenic potential after HBV genome integrated into host chromosome, leading to hepatocellular carcinoma.[3,4]

The diagnostic and prognostic precision for CHB were boosted by the advancement of laboratory assays for quantification of serum virological (HBV nucleic acids) and immunological (HBV antigens and their corresponding antibodies) indicators.[5] Serum biomarkers have been widely used to evaluate CHB, but they still have limitations.[6,7] The histopathological examination is still the gold standard for diagnosis and assessment of liver pathologies and guide for proper antiviral treatment in CHB patients.[8] The immunohistochemistry (IHC) of hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) could be used to evaluate HBV activities within the liver tissue.[9,10] However, the moderate relationship of IHC of HBsAg with viral replication and the lower positive rate of HBcAg limited their clinical value .[11]

In situ hybridization (ISH) assay has a greater sensitivity that allows detection and localization of specific nucleic acids in various samples using different labeling methods.[12,13] Previously, we have developed a highly sensitive and specific ISH assay for detecting and visualizing HBV deoxyribonucleic acid (DNA) together with viral antigens, and found that the distribution of HBV DNA is distinct from that of HBsAg.[14] A 3-stage model was proposed to describe the complex lifecycle of HBV within the liver lobule.[14,15] In the current study, we sought to explore the performance of ISH assay in a large group of real-world CHB patients and to further probe the distribution pattern of HBV DNA relative to liver fibrogenesis.

2. Material and methods

2.1. Patients

One hundred four patients without contraindications to percutaneous liver biopsy, hospitalized in Chinese People's Liberation Army (PLA) Diagnosis and Treatment Center for Liver Diseases, the 960th Hospital of Chinese PLA Joint Logistics Support Force, were enrolled in this study, all of whom underwent liver biopsy and histological examinations. Among them, 94 were CHB-patients with or without antiviral treatment. The other 10 patients had non-HBV-related liver diseases such as hepatitis C virus infection, liver injury without definite diagnosis etc. Among the CHB patients, 60 were hepatitis B e antigen (HBeAg)-positive and 34 were HBeAg-negative. The diagnosis criteria were in accordance with the standards elaborated in the European Association of Study of Liver Diseases 2017 Clinical Practice Guidelines on the management of CHB.[16]

2.2. Pathological examination and evaluation

Percutaneous liver biopsy was performed on the enrolled patients using a 16G disposable spring-loaded cutting needle under the guidance of ultrasonography. The obtained liver tissue specimens were fixed with 3.7% neutral formaldehyde, embedded in paraffin, and serially sectioned at 5 μm per slide. The paraffin-fixed tissue slides were routinely stained with hematoxylin and eosin, Masson trichrome and reticulin. The liver pathological diagnosis was reviewed by 2 experienced pathologists independently who were blinded to patients' identity and history. The grade of inflammation and stage of fibrosis were evaluated according to Scheuer scoring system.

IHC of HBsAg and HBcAg were performed using Leica BOND automatic stainer and were semiquantitatively evaluated by 2 independent observers. According to the proportion of immunolabeled cells of 0%, <10%, 10% to 20%, and >20% in liver sections, they were scored as 0, 1, 2, and 3, respectively.

2.3. Serum biomarker quantification

Liver enzymes (alanine transaminase [ALT], aspartate transaminase, [AST]), HBV serological markers (HBsAg, HBeAg, anti-HBeAg), and HBV DNA were detected within 7 days before liver biopsy. Liver enzymes were measured by BECKMAN COUTER Chemistry Analyzer AU5400 (BECKMAN COUTER, Japan). Measurement of HBV serological markers was performed using CHIVD Light Initiated Chemiluminescence Analyzer LiCA500 (CHIVD, China). The detection range of serum HBsAg is 0.05 to 250 IU/mL, and it was redetected with a sample dilution of 1:500 if the initial examination value exceeding the upper detection limit. Serum HBV DNA was quantified using hepatitis B viral DNA Quantitative Fluorescence Diagnostic Kit (Sansure, China) with a detection range of 5×10^2 to 2×10^9 IU/mL.

2.4. In situ hybridization

ISH of HBV DNA was performed using the ViewRNA ISH Tissue Assay (Thermo Fisher, Fremont, CA) as described in our previous study with some modifications.[14] Briefly, the tissue sections were routinely dewaxed and rehydrated in 100% ethanol, 50% ethanol and deionized water, followed with antigen retrieval, protease digestion and refixation with 4% formaldehyde for 5 minutes. The HBV DNA probe (VF6-20095) was designed to specifically target the minus strand sequence (nt2959-837) conserved from genotype A to D. After probe hybridization and signal amplification, sections were stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Roche) in developing solution at 37°C for 2 hours. The slides were counterstained with Sirius red for 5 minutes and washed with distilled water. Finally, sections were dehydrated and mounted in mounting medium. The ISH results were examined with a bright-field microscope and graded according to the proportion of HBV DNA positive hepatocytes, ranged from 0 to 3. In particular: 0 = no signal, 1 = spot positive signal, 2 = 0% to 10% positive hepatocytes, 3 = >10% positive signal. Slides were scanned by Panoramic Digital Slide Scanner. For quantitative analysis the HBV DNA-ISH positive rate, we processed the scanning section images with ImageJ and analyzed DNA ISH positive area ratio compared to the tissue section area. To analyze the spatial relationship between HBV DNA and collagen deposition, 12 patients with the HBV DNA-ISH score of 3 were selected, 30 sub-regions (100 × 100 pixels) from each whole tissue scan were sampled and analyzed by calculating the proportion of DNA ISH positive area and collagen deposition area with ImageJ. The aggregated data were visualized as dot plot and correlation analysis was performed (Spearman correlation).

2.5. Ethics statement

This study was conducted in compliance with the ethical guidelines of the 2013 Declaration of Helsinki and ethical approval was obtained from the independent ethics committee of the 960th Hospital of Chinese PLA Joint Logistics Support Force.

2.6. Statistical analysis

Statistics were analyzed using SPSS 23 (SPSS Inc., Chicago, IL) and GraphPad Prism 7.0 (Graph Pad Software Inc., San Diego, CA).

Table 1

Clinical and virological features of the patients with chronic HBV infection.

Features	HBeAg-positive (n = 60)	HBeAg-negative (n = 34)	$Z^*/\chi^{2\dagger}$	P
Gender (male: female)	46:14	26:8	0.02157†	.98
Age (yr; M, IQR)	43 (33-51)	44 (34-52)	−0.224*	.82
ALT (U/L; M, IQR)	85 (28-213)	35.5 (15-178)	2.164*	0.03
Serum HBV DNA (log10 IU/mL; M, IQR)	6.96 (4.57-8.02)	3.10 (2.70-4.97)	4.657*	<.0001
HBsAg IHC (0:1:2:3)	2:36:3:19	3:25:0:6	5.146†	.16
HBcAg IHC (0:1:2:3)	40:15:0:0	30:2:0:0	5.687†	.023
HBV DNA-ISH positive rate (%; M, IQR)	0.424 (0.01024–0.741)	1.499 (0.464–3.0025)	4.100*	<.0001
HBV DNA-ISH score (0:1:2:3)	16:16:17:11	25:7:1:1	22.590†	<.0001
Grade (1:2:3:4)	5:30:24:1	5:21:8:0	3.678†	.29
Stage (1:2:3:4)	4:26:12:18	5:10:10:9	3.479†	.32

ALT = alanine transaminase, HBV = hepatitis B virus, IHC = immunohistochemistry, IQR = interquartile range, ISH = in situ hybridization, M = median.

* Wilcoxon-rank sum test.

† Pearson χ^2 test.

Wilcoxon-rank sum test was employed to compare continuous variables between HBeAg-positive and HBeAg-negative groups.[17] The categorical variables between groups were analyzed with Pearson χ^2 test or Fisher exact test.[18] Spearman correlation analysis was employed to evaluate correlation between non-normally distributed continuous data or categorical variables.[19] Differences were considered statistically significant for *P* values less than .05 (2-tailed).

3. Results

3.1. Clinical and virological features of the patients with chronic HBV infection

A total of 94 CHB patients were categorized into HBeAg-positive (n = 64) and HBeAg-negative (n = 30) groups. The baseline patients characteristics are summarized in Table 1. No differences were found in the male-to-female ratios ($P = .98$) and age ($P = .82$) between these 2 groups. HBeAg-positive patients exhibited higher serum ALT level than HBeAg-negative group (median, 85 U/L vs 35.5 U/L, $P = .03$). The level of serum HBV DNA in HBeAg-positive patients was significantly higher than that in HBeAg-negative patients (median, 6.96 $\log_{10}$ IU/mL vs 3.10 $\log_{10}$ IU/mL, $P < .0001$).

3.2. HBV DNA-ISH signal pattern and its relationship with serum viral titre

The ISH assay enables visualization of HBV DNA within the livers of CHB patients and did not show observable signal in 10 cases of HBV negative controls (Fig. S1, Supplemental Digital Content, http://links.lww.com/MD2/A450 for 1 exemplary case). A score (from 0-3) for the ISH signal was given to each liver biopsy section. Among all included patients, the positive rate of HBV DNA, HBsAg-IHC, and HBcAg-IHC were 55.8%, 94.7%, and 19.5%, respectively. This ISH assay had higher positive rate in HBeAg-positive patients (44/60, 73.3%) than in HBeAg-negative group ((9/34, 29%, $P < .0001$). Similarly, HBcAg IHC presented 25% (15/60) positive rate in HBeAg-positive patients compared with 6% (2/30) in HBeAg-negative group ($P = .023$). There was no significant difference between the positive rate of HBsAg IHC in HBeAg positive and negative patients (58/60, 96.7% vs 31/34, 91.2%, $P = .16$).

In-depth analysis of the distribution and spatial localization in liver biopsy showed obvious difference between HBV DNA and HBsAg in most cases. As shown in Figure 1, HBV DNA signal was mostly distributed in the cytoplasm of the hepatocytes and showed a discrete lobular distribution as it was often concentrated in collagen fiber-rich areas (Fig. 1A) whereas the HBsAg was tended to be accumulated in areas with lower degree of collagen invasion (Fig. 1B) as shown in a HBeAg positive patient with viral load of 4.45×10^8 IU/mL. The segregated distribution of these 2 markers were also manifested in the whole tissue level. As shown in the contingency table created with HBsAg and HBV DNA, these markers are totally unrelated statistically ($P = 1.000$, Fisher exact test, Table S1, Supplemental Digital Content, http://links.lww.com/MD2/A451). By contrast, 3 in 38 (7.89%) of the HBV DNA-ISH negative cases were positive for HBcAg while in HBV DNA-ISH positive cases, 14 in 49 (28.6%) cases were positive for HBcAg ($P = .0272$, Fisher exact test, Table S2, Supplemental Digital Content, http://links.lww.com/MD2/A452). These results indicated that HBcAg IHC is linked with HBV DNA-ISH assay. In addition, IHC of HBcAg and HBsAg, due to the lower rate of HBcAg, no correlation was shown with each other ($P = 1.000$, Fisher exact test, Table S3, Supplemental Digital Content, http://links.lww.com/MD2/A453). This implies that only viral antigen staining may be not preferable to evaluate intrahepatic viral replication.

We then analyzed its correlation between HBV DNA-ISH and serum virological markers. Spearman rank correlation showed there was a robust positive correlation between HBV DNA-ISH signal and serum HBV DNA ($r_s = 0.6223$, $P < .0001$) (Fig. 2).

Next, comparison was performed between intrahepatic antigen accumulation and serum HBV DNA. The IHC signal of HBsAg showed marginal correlation with serum HBV DNA ($r_s = 0.24$, $P = .02$, Fig. 3B) whereas a much more significant correlation between HBcAg IHC and viral load was observed ($r_s = 0.35$, $P = .0008$, Fig. 3C). In comparison, HBV DNA-ISH score was highly correlated with serum HBV DNA ($r_s = 0.6993$, $P < .0001$, Fig. 3A), note that the HBV DNA-ISH graded score transformed from its positive signal that was consistent with the IHC score.

3.3. Intimate relationship between intrahepatic HBV DNA and fibrogenesis

It has been shown in Figure 1 that compared with HBsAg, HBV DNA seemed to be spatially close to collagen fibers, which prompts us that HBV plays a role in inducing the expression of

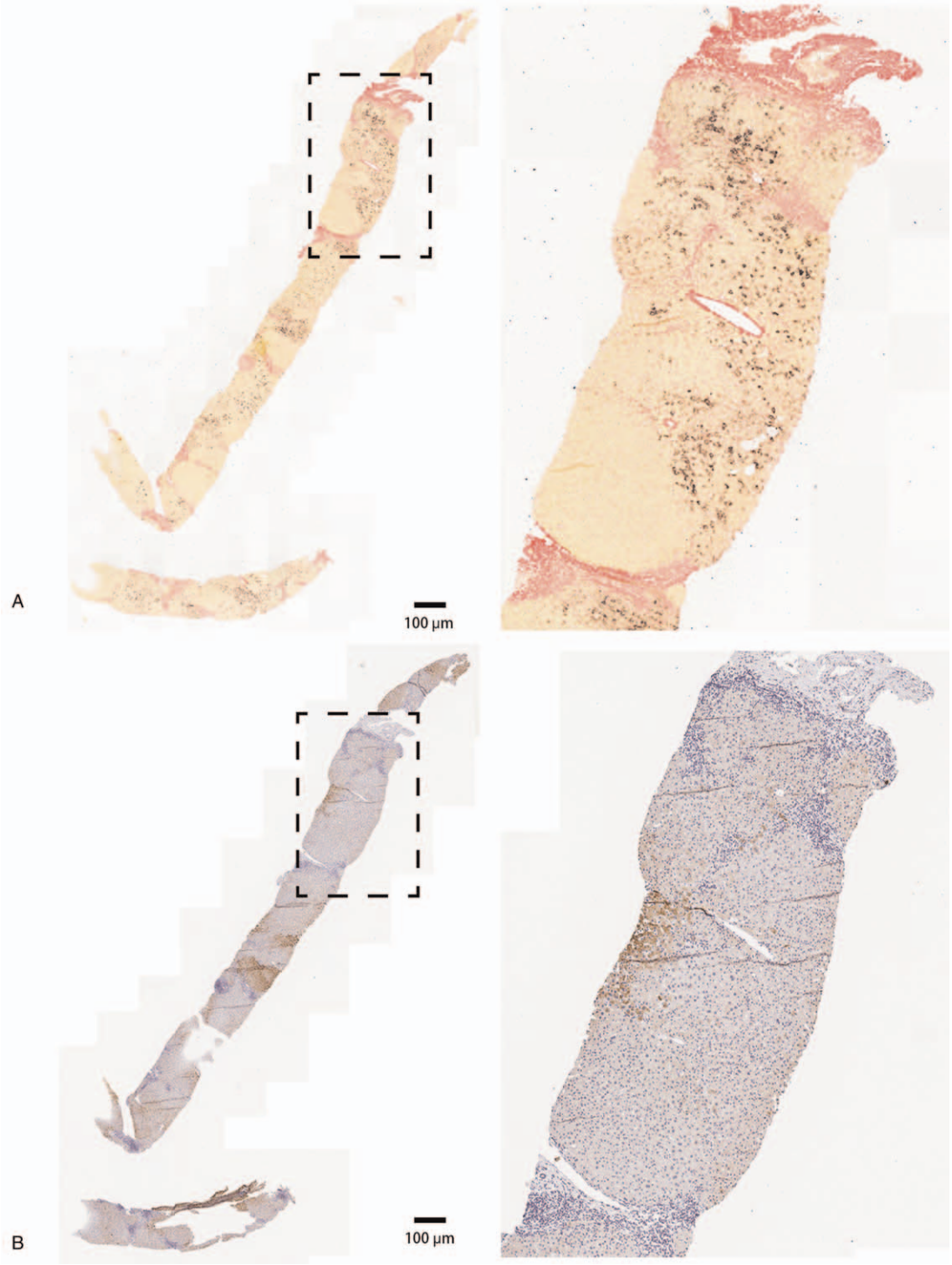

Figure 1. Comparison of in situ hybridization of HBV DNA and immunohistochemistry of HBsAg in a HBeAg-positive chronic hepatitis B patient with HBV DNA of 4.45×10^8 IU/mL, ALT of 65 U/L and its HBsAg level was 850 IU/mL. The liver biopsy section scanning image were shown (A and B) and representative areas were enlarged in the right panel. Original magnifications were ×25 and ×100. ALT = alanine transaminase, HBV = hepatitis B virus.

collagen in the liver. To further validate this observation, we examined more cases with medium to advanced liver fibrosis. Indeed, HBV DNA positive cells were repeatedly found to be in close contact with portal areas invaded by collagen (Fig. 4A). In addition, in cases with advanced fibrosis (S4), HBV DNA positive cells were found to be surrounded by collagen fibers intruded into the parenchyma (Fig. 4B), some of which were besieged in the pseudo-lobule (Fig. 4C). We tested the statistical significance of the spatial correlation between these 2 markers by sampling 30 independent sub-regions (100 × 100 pixel) from each of the 12 cases with HBV DNA-ISH score of 3. The percentage of HBV DNA and collagen fiber for each subregion was quantified and

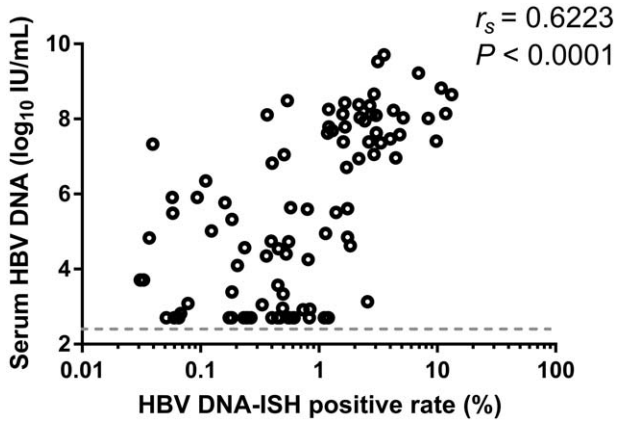

Figure 2. Correlation analysis between HBV DNA-ISH positive rate and serum HBV DNA load. Spearman correlation coefficient and *P* value were shown in the graph. HBV = hepatitis B virus, ISH = in situ hybridization.

the aggregate data were analyzed. As shown in Figure 5, the strong correlation ($r_s = 0.3788$, $P < .0001$), between these 2 markers supported their spatial relationship. These data further suggested the possible mechanistic links between the DNA-rich stage of HBV infection and liver fibrogenesis.

4. Discussion

Chronic HBV infection manifests with highly variable diseases ranging from immune tolerance to severe necroinflammation depending on an array of viral and host factors, which till now are still not fully understood. Detection of the presence and activity of HBV are the foundations of disease diagnosis and management. Although serum biomarkers such as viral load, HBsAg, and HBeAg titer are convenient to use,[20] they have a series of limitations in guiding appropriate diagnosis and treatment. For instance, some CHB patients exhibit less than 2× ULN of ALT level but have already developed serious liver disease. Liver pathology thus become indispensable for some categories of patients.[21]

Apart from conventional histology examinations, molecular pathology for HBV has mainly restricted to immune detection of viral surface and core antigens. In immune-tolerant patients and carriers, intrahepatic HBsAg (predominantly large hepatitis B surface antigen, LHBsAg) accumulation in the cytoplasm results in a ground-glass appearance.[22,23] Its intrahepatic abundance, however, was not tightly associated with either circulating S antigen or viral load.[24] IHC of core antigen usually exhibits a nuclear localization and is much less sensitive than that of HBsAg.[25] Although ISH of HBV DNA has been reported in as early as 1980s, its clinical use has been quite restricted. We previously developed a sensitive ISH assay for HBV DNA and used it in conjunction with immune detection of surface and core antigen. We unexpectedly uncovered a highly complex distribution indicating the heterogeneous virological states at single-cell level.[14] In order to further evaluate the usability of our ISH assay, we performed a real-world study recruiting CHB patients in various stages of disease irrespective of their prior treatment history. HBV DNA-ISH, IHC of surface and core antigen together with serum biomarkers were analyzed and compared. These data provided some key information as follows.

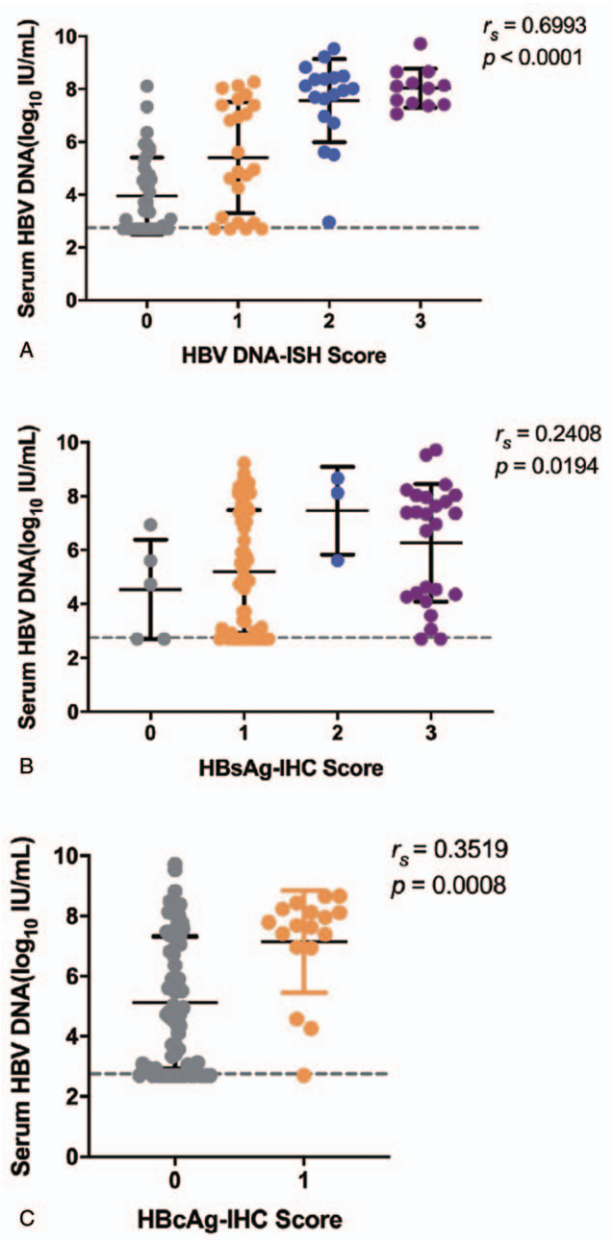

Figure 3. Statistical analysis between intrahepatic virologic examinations and serum HBV DNA load. The level of serum HBV DNA was grouped according to the ISH score for HBV DNA (A), IHC score for HBsAg (B), and HBcAg (C). HBV = hepatitis B virus, IHC = immunohistochemistry, ISH = in situ hybridization.

Firstly, we observed distinct distribution patterns of HBV DNA and HBsAg as shown in the head-to-head comparison of the whole liver section. The ISH of HBV DNA showed a lower sensitivity than IHC of HBsAg (55.8% vs 94.7%), it might be the result of efficient antiviral therapy.[26] Besides, IHC of HBcAg has a much lower positive rate, it further confirmed the limitation of immunohistology of viral antigen.

Second, we evaluated the clinical relevance of HBV DNA in situ with serum virological markers. The result showed a highly significant positive correlation of HBV DNA-ISH with serum HBV DNA in CHB. In comparison, the degree of correlation between IHC scores and serum viral load was marginal.

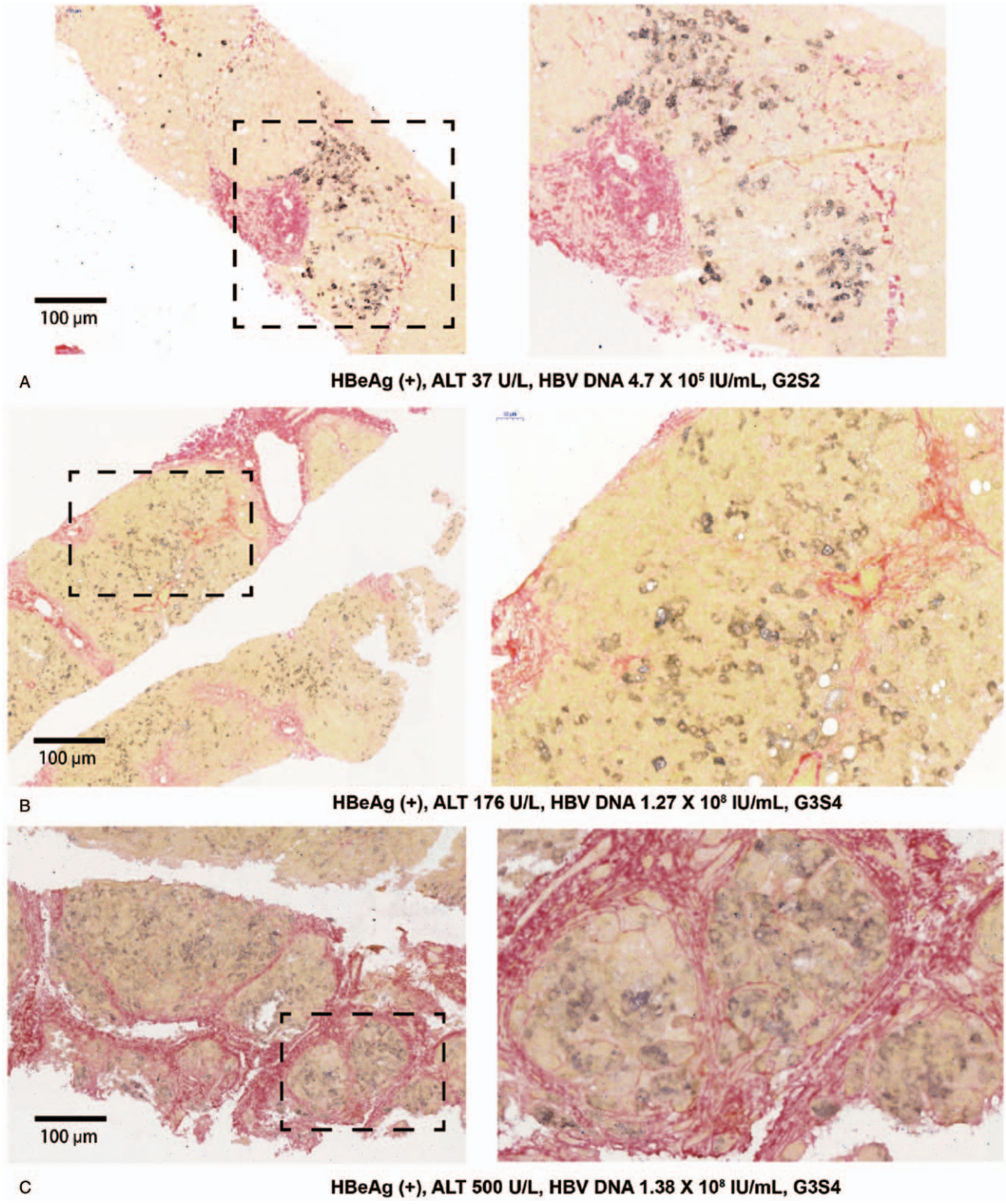

Figure 4. Representative images of the HBV DNA signal in different stages of CHB. Basic clinical laboratory parameters (HBeAg status, ALT level, serum HBV DNA load) and Scheuer score were listed under each panel. Original magnifications were ×100 and ×400. ALT = alanine transaminase, CHB = chronic hepatitis B, HBV = hepatitis B virus.

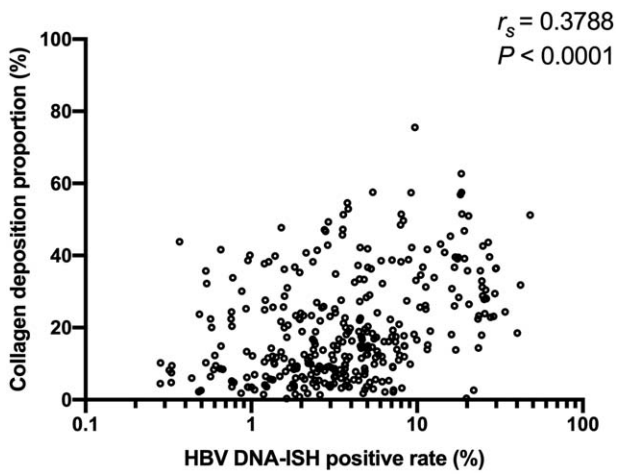

Figure 5. HBV DNA ISH signal and collagen fiber are spatially correlated. Twelve cases with HBV DNA ISH score of 3 were analyzed. Thirty subregions (100 × 100 pixel) were randomly picked from each of the whole tissue scan image. The positive rate of HBV DNA and collagen fiber were analyzed for each subregion. The aggregate data (n=360) were plotted and analyzed for correlation. HBV = hepatitis B virus, ISH = in situ hybridization.

Lastly, the distribution of HBV DNA in the context of collagen fiber showed a close spatial relationship suggestive of a functional link. This double staining assay informs us of a specific spatial relationship among virologic and histologic events and give us a unique view of liver disease progression.

This study also has limitations. Patients with current antiviral therapy or other comorbidities were included which may weaken statistical significance. No follow-up analysis was done to monitor the intrahepatic dynamics of viral activity.

In summary, this optimized ISH of HBV DNA assay enables us to sensitively and specifically visualize the virological activities within liver tissues and could be used as indicators of hepatitis activity. Moreover, this methodology maybe further used to evaluate the response of antiviral therapies by demonstrating the elimination of HBV DNA in clinical biopsy specimens from successfully treated patients, it has the potential to be applied as a routine pathological examination complementary to conventional IHC staining in clinical practice.

Author contributions

XZ, QS conceived the study. CL, WZ, BS, GaC collected the samples and performed the experiments. YZ, YA and MS provided technical support. XZ, YF and QS analyzed the data. CL and XZ drafted the manuscript.

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Writing – review & editing: Chang Li, Qinghua Shang, Xiaonan Zhang.

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