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Association between intrahepatic cccDNA and the severity of liver inflammation in chronic hepatitis B virus infection patients

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Background and aims: This research aimed to examine the association between hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) and liver inflammation in chronic hepatitis B (CHB) infection patients.

Methods: From August 2013 to June 2022, CHB patients at Hebei Medical University Third Hospital (Hebei, China) were recruited. Intrahepatic cccDNA was quantified and its association with liver inflammation was analyzed. Liver inflammation was assessed using the Ishak-modified histological activity index (HAI). Biochemical and viral indicators as well as hepatic inflammation biomarkers were monitored.

Results: In total, 55 CHB patients were enrolled. The average HBV-cccDNA level was markedly elevated in HBeAg+ patients compared to HBeAg patients. Intrahepatic cccDNA levels differed significantly in different liver inflammation groups and showed a positive correlation with the HAI score for hepatic inflammation.

Conclusion: HBV-cccDNA level was associated with liver inflammation.

KEYWORDS

covalently closed circular DNA (cccDNA), inflammation, liver, HBV, chronic hepatitis B

1 Introduction

Hepatitis B virus (HBV) is a significant contributor to viral hepatitis, which affects over 257–291 million individuals globally. The interplay between HBV viral propagation and the immune system's reaction gives rise to various health consequences, from liver inflammation to hepatocellular carcinoma (HCC) and cirrhosis (1). HBV-cccDNA formation is critical for the virus's life cycle and is crucial in the persistence and recurrence of infection. Hence, intrahepatic HBV-cccDNA acts as an essential biomarker of disease progression and is widely utilized to assess the effectiveness of antiviral treatments and establish treatment goals (2–6).

The host anti-HBV-specific immune response can control infection by clearing infected hepatocytes. However, the virus can weaken and deplete the host's specific

antiviral cellular immune system by continuously expressing high levels of hepatitis-B-e-antigen (HBeAg) and hepatitis-B-surface-antigen (HBsAg), making it ineffective in clearing infected hepatocytes. In clinical practice, the negative conversion of HBeAg commonly signifies partial immune control over chronic hepatitis B (CHB) infection. Therefore, patients are assigned to HBeAg+ chronic HBV group, HBeAg+ CHB group, HBeAg chronic HBV infection group, and HBeAg CHB group according to their HBeAg status and severity of liver inflammation. The progression of persistent HBV relies on the interplay between the virus and host immunity. However, due to the lack of simple and effective host-specific immune evaluation indicators for HBV, the dynamic changes of host immune factors in persistent HBV and their role in disease progression remain largely unknown. Previous studies have shown that host immune activation against HBV can effectively suppress viral replication by targeting infected liver cells, but this response is typically seen in the early HBV stage in HBeAg+ patients with high levels HBV-DNA levels (7). HBV can undergo spontaneous reactivation or reactivate in response to immunosuppressive or anti-inflammatory treatments (8). The existing nucleos(t)ide analogs (NAs) inhibit the replication of cytoplasmic HBV; however, they do not address cccDNA and are not curative treatments (9). Magri et al. (10) revealed a gene signature related to immune responses involved in HBV-cccDNA transcription. In addition to aspartate transaminase (AST) and ALT, serum HBsAg level was found to correlate with the grade of inflammation in HBeAg+ CHB patients prior to NA treatment. Additionally, combining HBsAg with AST demonstrated outstanding diagnostic performance for predicting severe inflammation (11).

Therefore, this research aimed to evaluate the changes in HBV-cccDNA levels of CHB patients receiving liver biopsy based on their HBeAg status, liver function, liver tissue inflammation activity, viral load, and cccDNA levels.

2 Materials and methods

2.1 Objects

From August 2013 to June 2022, 55 patients with CHB at Hebei Medical University Third Hospital (Hebei, China) were recruited in this study. Persistent HBV patients receiving liver biopsy and screening for serum biochemical and viral indicators were also included. The following criteria were used for inclusion: (1) age 18–65 years; (2) the serum level of HBsAg was detected in the last 6 months; (3) the patient provided consent for a liver biopsy and testing of serum biochemical and viral indicators; and (4) informed consent in writing was provided for treatment and participation in this research. The following criteria were used for exclusion: (1) the patient had previously undergone antiviral therapy for HBV; (2) co-infection with other viruses, including HCV, HDV, and HIV; (3) diagnosed with compensated/decompensated cirrhosis or HCC; (4) autoimmune hepatitis, primary biliary cirrhosis, alcoholic/non-alcoholic liver diseases; and (5) coexisting conditions, or severe metabolic imbalance or psychiatric disorders. This research was granted approval from the Ethics Committee of the same institute.

2.2 Data collection

Demographic and laboratory parameters including age, gender, red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (HGB) level, platelet (PLT) count, and the levels of alanine aminotransferase (ALT), AST, alkaline phosphatase (ALP), albumin (ALB), total bilirubin (TBIL), glutamyl transpeptidase (GGT), HBsAb, HBsAg, HBcAb, HBeAg, HBeAb, and HBV-DNA were obtained retrospectively from the electronic medical records system.

2.3 Assessment of liver inflammation

Liver biopsies were conducted through standard procedures. Ultrasound-guided liver biopsy was conducted based on the standard procedure (12). At least 2 pieces with 2.0 cm length were collected to ensure the presence of 11 portal tracts for histological evaluation. Two pathologists independently evaluated the biopsy specimens, blinded to both the biopsy timing and clinical information. In cases of inconsistency, both pathologists reexamined the samples to reach a consensus. Liver inflammation was assessed using the Ishak-modified histological activity index (HAI) (13, 14).

2.4 Detection of intrahepatic HBV DNA and cccDNA levels

About 30 μm formalin fixed paraffin-embedded liver biopsy tissue in sections of 6 μm each was used for DNA extraction. To prevent contamination, disposable tweezers, brush, and interleaver were used and sectioning blades were carefully cleaned with 70% ethanol after every sampling. Genomic DNA and intracellular-free microchromosomal DNA were isolated using the QIAamp-DNA-Mini-Kit in accordance with the manufacturer's guidelines (QIAGEN, Germany). PSAD was utilized for digestion of HBV double-stranded DNA, relaxed circular DNA, and single-stranded DNA (Epicentre, USA). Subsequently, cccDNA-selective amplification was carried out using the rolling-circle-amplification (RCA) technique with Phi29 (New England Biolabs, USA). RCA products were further amplified and quantified via TaqMan real-time PCR, employing probes that target the unfilled portions of the viral genome along with cccDNA-specific primer pairs. Notably, Phi29 and PSAD were unnecessary for detecting total HBV-DNA. The cell count was determined using probes and primers as a standard control, specifically DNA fragments of human β-actin. The LLOQ was 0.01 copies per cell.

2.5 Statistical analysis

All statistical tests were conducted using GraphPad Prism v8.0, MedCalc v15.0, and SPSS v26.0. Continuous data are presented as mean ± SD or median (IQR). They were compared using the Kruskal–Wallis test. Categorical data were examined using the χ^2 test. They are presented as numbers (percentages). The correlations between two parameters were determined

TABLE 1 Baseline characteristics.

	HBeAg-negative patients (*n*=26)	HBeAg-negative patients (*n*=29)	*t*	*p*
Sex (male/female)	21/7	17/10	0.933	0.334
Age (years)	37.11 ± 12.73	39.81 ± 10.46	−1.121	0.262
WBC (10^9/L)	5.45 ± 1.37	6.02 ± 1.35	−1.704	0.088
HGB (g/L)	154.00 (136.00, 162.00)	152.90 (129.60, 160.00)	−0.459	0.647
PLT (10^9/L)	189.46 ± 70.90	209.28 ± 58.20	−1.107	0.268
Albumin (g/L)	46.45 (40.48, 48.68)	47.40 (44.15, 4872)	−1.136	0.256
ALT (U/L)	51.50 (29.00, 131.75)	27.00 (22.00, 50.00)	−2.467	0.014
AST (U/L)	32.00 (21.40, 71.50)	25.00 (20.00, 30.00)	−1.912	0.056
Total bilirubin (μmol/L)	17.52 (12.64, 22.94)	14.28 (12.06, 22.35)	−0.892	0.372
ALP (U/L)	69.50 (52.42, 88.00)	70.00 (48.00, 90.00)	−0.044	0.965
GGT (U/L)	43.50 (27.50, 68.50)	25.00 (16.00, 44.00)	−2.90	0.022
HBsAg (log10 IU/mL)	3.89 (3.51, 4.50)	2.97 (1.97, 3.72)	−3.862	0.000
HBeAg (log10 IU/mL)	2.43 (1.15, 3.06)	−0.40 (−0.49, −0.37)	−6.186	0.000
Serum HBV-DNA (log10 IU/mL)	7.16 (4.71, 8.00)	3.14 (2.09, 3.94)	−4.694	0.000
Intrahepatic HBV-DNA (log10 IU/mL)	1.83 (0.99, 2.83)	−2.00 (−2.00, 0.52)	−4.306	0.000
Intrahepatic cccDNA (copies/cell)	0.53 (0.04, 3.25)	0.01 (0.01, 0.05)	−3.587	0.000

HBeAg, hepatitis B e antigen; WBC, white blood cell; RBC, red blood cell, HGB, hemoglobin; PLT, platelet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, glutamyl transpeptidase; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; cccDNA, covalently closed circular DNA.

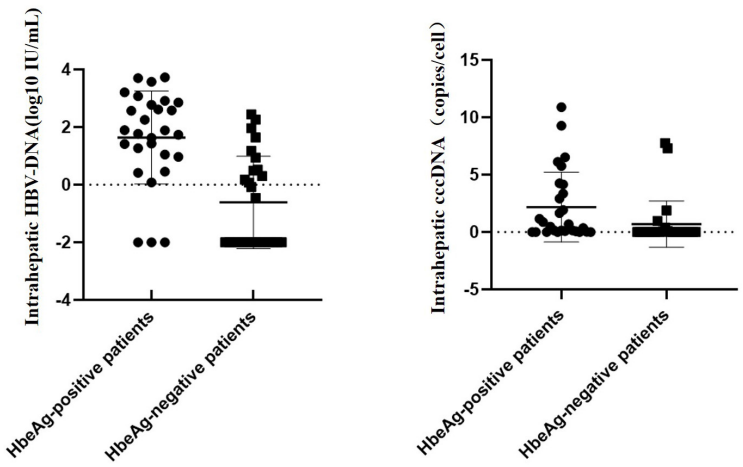

FIGURE 1
Hepatitis B virus-DNA and cccDNA levels in HBeAg+ and HBeAg patients.

using Pearson's correlation. Two-tailed tests of significance were conducted, and $p < 0.05$ was regarded as statistically significant.

3 Results

3.1 Baseline features

In total, 55 patients were included between August 2013 and June 2022. These patients were categorized into two groups according to their HBeAg status. No remarkable differences were observed between the two groups regarding sex, age, AST, total bilirubin, and albumin between the HBeAg and HBeAg+ groups. However, obvious differences were noted between the two groups regarding AST, GGT, serum HBV-DNA, serum HBsAg, intrahepatic HBV-DNA, and intrahepatic HBV-cccDNA levels (Table 1).

3.2 Variation in HBV-cccDNA and serum-based viral indicators

When stratifying patients by the HBeAg status, the mean HBV-cccDNA and intrahepatic HBV-DNA levels of patients with

TABLE 2 Expression levels of cccDNA in different inflammation groups.

	Group A (n=21)	Group B (n=34)	t	p
Sex (male/female)	17/4	21/13	2.238	0.135
Age (years)	39.00 (31.00, 45.50)	37.00 (30.75, 42.00)	−0.217	0.828
WBC (10^9/L)	5.67 ± 1.37	5.76 ± 1.41	−0.257	0.797
HGB (g/L)	152.00 (132.50, 156.50)	154.00 (134.75, 161.80)	−0.639	0.523
PLT (10^9/L)	206.20 ± 67.67	187.70 ± 60.87	−1.100	0.271
Albumin (g/L)	46.94 ± 3.52	42.85 ± 6.82	−1.784	0.074
ALT (U/L)	92.00 (42.50, 187.50)	28.00 (22.75, 46.00)	−4.177	0.000
AST (U/L)	54.00 (32.00, 85.0)	22.50 (20.00, 27.00)	−4.430	0.000
Total bilirubin (μmol/L)	22.53 (13.79, 25.78)	14.03 (12.02, 20.00)	−2.460	0.014
ALP (U/L)	68.00 (48.00, 104.00)	71.00 (52.43, 88.50)	−0.250	0.802
GGT (U/L)	59.00 (35.00, 83.50)	23.50 (16.00, 37.75)	−4.150	0.000
HBsAg (log10 IU/mL)	3.89 (3.18, 4.21)	3.49 (2.48, 3.84)	−1.625	0.104
HBeAg (log10 IU/mL)	2.36 (−0.36, 3.04)	−0.36 (−0.43, 1.14)	−2.461	0.014
Serum HBV-DNA (log10 IU/mL)	6.58 (3.39, 7.97)	3.68 (2.42, 5.16)	−2.384	0.017
Intrahepatic HBV-DNA (log10 IU/mL)	1.64 (0.46, 2.59)	0.13 (−2.00, 1.91)	−2.181	0.029
Intrahepatic cccDNA (copies/cell)	0.69 (0.20, 5.19)	0.01 (0.01, 0.41)	−2.967	0.003

HBeAg, hepatitis B e antigen; WBC, white blood cell; RBC, red blood cell, HGB, hemoglobin; PLT, platelet;ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, glutamyl transpeptidase; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; cccDNA, covalently closed circular DNA.
Group A: patients with inflammation (serum ALT level above 2ULN); group B: patients who did not experience inflammation (serum ALT level below 2ULN).

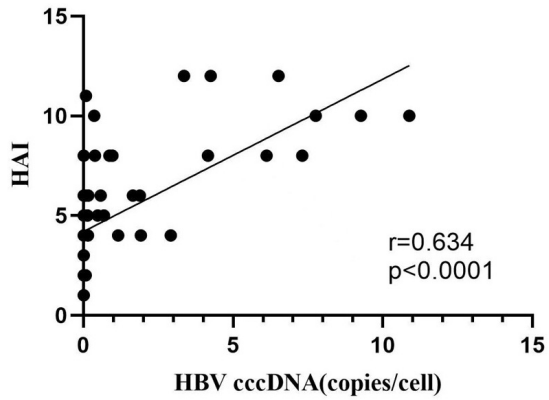

FIGURE 2
Correlation between HBV cccDNA and HAI.

HBeAg+ were remarkably increased compared to those with HBeAg (0.53 vs. 0.01 log10 copies/cell, 1.83 vs. 2 log10 IU/mL, $p < 0.001$) (Figure 1).

3.3 HBV-cccDNA and hepatic inflammation

Fifty-five patients with ALT < 2 ULN were categorized into two groups according to the severity of hepatic inflammation. Twenty-one patients with liver inflammation (ALT more than 2 ULN) were allocated into group A. Thirty-four patients without liver inflammation (ALT less than 2 ULN) were allocated into group B. Age, sex, and ALB did not markedly vary between the two groups. There were obvious differences in AST, ALT, GGT, total bilirubin, serum HBsAg, serum HBeAg, intrahepatic HBV-DNA levels, serum HBV-DNA, and intrahepatic HBV-cccDNA levels between the two groups (Table 2). Besides, logistic regression analysis revealed positive correlations between HBV-cccDNA levels and liver inflammation HAI scores (Figure 2).

3.4 Correlations between HBV-cccDNA and viral indicators

Pearson correlation analysis indicated that intrahepatic cccDNA level was positively correlated with HBsAg, HBeAg, HBeAb, and serum HBV-DNA levels (r = 0.312, 0.314, 0.295, 0.403, $p < 0.05$). Intrahepatic HBV-DNA level was positively correlated with HBsAg, HBeAg, HBeAb, serum HBV-DNA, and intrahepatic HBV-cccDNA levels (r = 0.618, 0.700, 0.625, 0.815, and 0.501, $p < 0.001$) (Figure 3 and Table 3).

4 Discussion

Our findings indicated that the HBV-cccDNA level was markedly higher in HBeAg+ patients than in HBeAg patients. The reduced viral load in serum during the HBeAg phase seems to be associated with mechanisms other than merely the repression of intracellular HBV-cccDNA, and the replicative effectiveness of HBV is also involved in this phenomenon (15). Furthermore, the reduced levels of intracellular pregenomic RNA also indicate the suppression of serum HBV-DNA in HBeAg

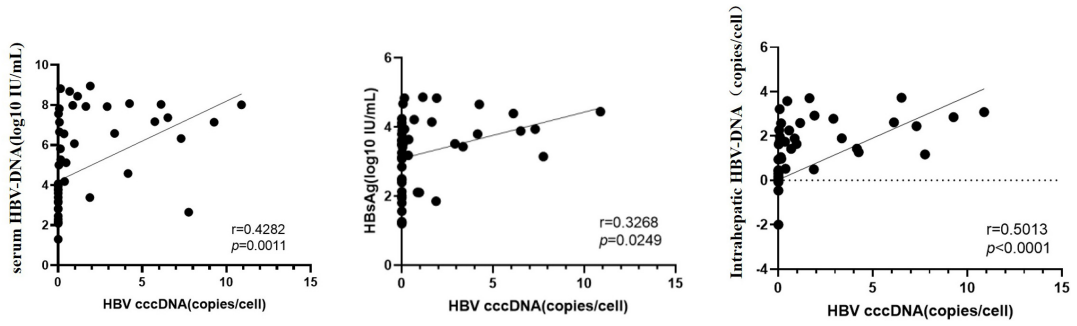

FIGURE 3
Correlation between HBV cccDNA and virological markers.

TABLE 3 Correlation between HBV-DNA, cccDNA and liver function, virological markers.

	Intrahepatic HBV-DNA (copies/cell)		Intrahepatic cccDNA (copies/cell)	
	r	*p*	*r*	*p*
ALT (U/L)	−0.109	0.428	0.001	0.996
AST (U/L)	−0.138	0.323	−0.007	0.962
HBsAg (log10 IU/mL)	0.618	<0.001	0.312	0.033
HBeAg (log10 IU/mL)	0.700	<0.001	0.314	0.030
HBeAb (log10 IU/mL)	0.625	<0.001	0.295	0.042
HBcAg (log10 IU/mL)	0.136	0.355	0.103	0.486
serum HBV-DNA (log10 IU/mL)	0.815	<0.001	0.403	0.002
Intrahepatic cccDNA (copies/cell)	0.501	<0.001	—	—

patients. Additionally, the serum HBV-DNA concentration is affected by the amount of intracellular HBV-cccDNA and the virus's replication efficiency (16).

Our results indicated that serum viral markers positively related to intrahepatic HBV-cccDNA levels in HBsAg, HBeAg, HBeAb, and HBV-DNA, which are in agreement with prior research (17–19). For instance, Thompson et al. (18) found that numerous hepatocytes stained positive for HBsAg in HBeAg+ patients with suppressed viral propagation, suggesting that the link between HBV replication and HBsAg production weakens during the HBeAg stage. This is likely due to HBsAg being produced from sources other than intracellular HBV-cccDNA. Additionally, prior research has reported a correlation between serum HBsAg levels and cccDNA copy number (20).

In our study, intrahepatic HBV-cccDNA levels, along with HBV-DNA viral load, HBeAg, and HBsAg titers, were substantially higher in HBeAg+ patients than in HBeAg patients, likely due to increased viral protein synthesis and release in individuals with elevated intracellular HBV-cccDNA levels. Such immune activation may worsen inflammation, resulting in elevated serum ALT concentrations. Intrahepatic HBV-cccDNA level was evaluated through liver biopsies and histological assessment. This is usually accompanied by hepatocyte damage, hepatic flares, and histologic changes. HBV-cccDNA transcribes mRNA encoding HBV-specific proteins and can recruit and contribute to inflammatory cells that cause liver injury (21). The findings indicated that elevated intrahepatic HBV-cccDNA level could elevate the likelihood of hepatic inflammation. More research is needed to confirm HBV-cccDNA as a viral indicator for patients potentially requiring further outpatient support. Currently, there is controversy regarding the association between intrahepatic HBV-cccDNA and histopathological liver inflammation (22–25). Some investigators have found no significant association between HBV-cccDNA levels and hepatic inflammation grade in HBeAg patients, whereas other groups report that HBeAg patients with significant hepatic inflammation show elevated intrahepatic HBV-cccDNA levels. Magri et al. (10) showed that transcripts derived from cccDNA are linked to biomarkers of hepatic inflammation. The inflammatory hepatic transcriptome analysis indicated that 24 genes are remarkably correlated with cccDNA transcriptional activity (10). The study identified a gene signature related to the immune response associated with HBV-cccDNA transcription.

Herein, we prospectively examined the association between intracellular HBV-cccDNA content and hepatic inflammation in patients. HBV-cccDNA can be used as a biomarker for assessing liver disease severity in clinical practice. However, further research is warranted to delineate the immune pathways controlling viral replication.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Hebei Medical University Third Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SZ: Methodology, Writing – original draft, Data curation, Formal analysis, Project administration, Supervision, Writing – review & editing. CD: Methodology, Project administration, Writing – original draft. CC: Methodology, Writing – original draft. JZ: Methodology, Writing – review & editing. JL: Data curation, Methodology, Writing – review & editing. XZ: Data curation, Methodology, Writing – original draft. WR: Investigation, Methodology, Writing – original draft. YZ: Investigation, Methodology, Writing – original draft. YN: Methodology, Writing – original draft, Formal Analysis, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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