

**CORRELATION BETWEEN INTRAHEPATIC cccDNA
AND SERUM HBV pgRNA IN TREATMENT-NAÏVE CHRONIC
HEPATITIS B PATIENTS**

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Abstract

Objectives: To evaluate the correlation between intrahepatic covalently closed circular DNA (cccDNA) and serum HBV pregenomic Ribo nucleic acid (pgRNA) in treatment-naïve chronic hepatitis B (CHB) patients. **Methods:** A total of 105 treatment-naïve CHB patients from the Department of Infectious Diseases, Military Hospital 103, Vietnam Military Medical University, were enrolled from 2017 to 2020. Real-time RT-PCR methods were used to quantify serum HBV pgRNA and intrahepatic cccDNA. Clinical and histopathological assessments were also performed. The patients were categorized into two groups: HBeAg-positive CHB (n = 59) and HBeAg-negative CHB (n = 46). Statistical analyses were conducted using Medcalc 20.019. **Results:** In treatment-naïve patients, the average concentration of cccDNA and HBV RNA were 1.61 ± 0.40 log₁₀ copies/cell and 4.88 ± 1.65 log₁₀ copies/mL, respectively. A positive linear correlation was observed between HBV pgRNA and cccDNA, especially in the overall group and the HBeAg-negative subgroup, as well as in patients with HAI score ≥ 9 and Fibrosis score ≥ 3 ($r = 0.49$, $r = 0.56$, and $r = 0.57$; $p < 0.01$, respectively). **Conclusion:** The study findings indicate a positive linear correlation between intrahepatic cccDNA and serum HBV pgRNA in CHB patients. HBV pgRNA can serve as a reliable marker in the management of CHB patients.

Keywords: Chronic Hepatitis B (CHB); HBV pregenomic RNA; cccDNA.

INTRODUCTION

WHO (2023) estimates that 296 million people were living with CHB infection in 2019, with 1.5 million new infections each year. Hepatitis B resulted in an estimated 820,000 deaths,

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mostly from cirrhosis and hepatocellular carcinoma (primary liver cancer) [1]. In the life cycle of HBV, HBV cccDNA within liver cells serves as the crucial template for HBV RNA coding, playing a pivotal role in virus replication, persistent infection, and recurrence. While first-line antiviral agents like nucleos(t)ide analogues (NAs) efficiently inhibit HBV replication and control disease progression in most patients, they rarely lead to the complete elimination of chronic HBV infection due to their limited effect on cccDNA, the primary cause of viral persistence [2]. Intrahepatic cccDNA monitoring, which is essential for assessing treatment efficacy, is challenging in clinical practice due to the need for liver biopsy, inter-observer variability, and potential complications. Traditional serum markers like quantitative HBsAg and HBV DNA have been proposed to reflect the intrahepatic cccDNA profile; however, their correlations are insufficient for widespread clinical application.

Therefore, there is a need for surrogate markers to assess intrahepatic cccDNA transcriptional activity. HBV pgRNA, derived solely from intrahepatic cccDNA, offers a promising alternative as its quantification remains unaffected by viral antigens or antibody complexes. Consequently, serum HBV pgRNA levels can more accurately reflect

intrahepatic cccDNA transcriptional activity. While serum HBV pgRNA quantification has been proven useful in managing chronic HBV infection, especially in predicting antiviral efficacy, viral rebound after drug withdrawal, and predicting viral resistance, there is currently a lack of corresponding data on baseline serum HBV pgRNA levels before treatment, particularly in Vietnam.

Hence, in this cross-sectional study, we aimed to: *Determine the correlation between intrahepatic cccDNA and serum HBV pgRNA in treatment-naïve CHB patients.* The results of this research could contribute valuable insights into the potential clinical significance of serum HBV pgRNA as a marker for monitoring CHB patients.

MATERIALS AND METHODS

1. Subjects

A total of 105 treatment-naïve CHB patients were enrolled in this study. These patients received treatment at the Department of Infectious Diseases, Military Hospital 103, Military Medical University, from 2017 to 2020.

* *Inclusion criteria:* Based on the Guidelines for the diagnosis and treatment of CHB issued by the Ministry of Health in 2014, including patients diagnosed with CHB who had not received nucleos(t)ide analogues (NAs) treatment.

* *Exclusion criteria:* Encompassed pregnant and lactating women; individuals < 18 or > 70 years of age; patients with co-infection of human immunodeficiency virus (HIV) or hepatitis C virus (HCV); patients who refused liver biopsy or did not comply with treatment.

2. Methods

* *Study design:* A cross-sectional study was conducted. Liver tissues and blood samples were collected from the patients. Liver biopsy, serum alanine aminotransferase (ALT), and hepatitis B e antigen (HBeAg) were routinely measured or detected at Military Hospital 103. Histopathological results were performed at the Pathology Department, Military Hospital 103, using the histological activity index (HAI) score scale, with subgroups categorized as < 9 points and ≥ 9 points. The classification of liver fibrosis based on histopathology examination was as follows: Mild and moderate cirrhosis for scores of $F < 3$ and severe cirrhosis for scores of $F \geq 3$.

* *Quantification of serum HBV pgRNA levels:*

The quantification of HBV pgRNA was performed by a one-step RT-qPCR that carried out 190 in 40 μ L reaction mix, which contains 8 μ L of 5x HTOne Ultra RT-qPCR Probe master mix (HT

Biotec, Vietnam), 2 μ L HBV common forward primer (HBV-F primer: 10 μ M), 2 μ L HBV RNA reverse primer (HBV-RT primer: 10 μ M), 0.8 μ L HBV common probe (HBV probe: 10 μ M), 15.2 μ L DEPC water, and 12 μ L template. The amplifications were performed in the Rotor-Gen Q (QIAGEN, Germany), and the thermal cycling program included 10 min at 50°C for reverse transcription, 15 min at 95°C for initial denaturation, 45 cycles of 15s at 94°C for denaturation, 30s at 63°C for annealing, and 30s at 72°C for extension. A positive control, a no-RT control, and a non-template control (NTC) were included in each PCR assay tested. Primers and probes for quantification of HBV pgRNA were designed on the conserved region of the S gene. The reverse primer for quantification of HBV pgRNA contained a 10-nucleotides sequence at the 3' end that was specific for the HBV genome and further contained a 5' tail of unrelated sequence (underlined). The primers and probe used were specific to the conserved region of the S gene (forward primer: 5'- GCCAAAATTCGCAGTCCC-3'; reverse primer: GCCACTCATCAGT-CAGCAGGATG; probe: FAMCGC-TGGATGTGTCTGCGGCGT-BHQ1). The limit of quantification of this assay was 100 copies/mL [3]. For statistical

analysis, 100 copies/mL (2 log copies/mL) was recorded as the value for serum samples with HBV pgRNA was below LOD or not detected [4].

* *Quantification of intrahepatic cccDNA levels:*

Intrahepatic HBV DNA was extracted from FFPE liver biopsy tissue using the QIAamp FFPE DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany). The amplification contained two steps as follows: (1) 10 μ L of PSAD digested DNA was mixed with primers at a concentration of 0.5 μ mol/L each and 1 μ L reaction buffer; the DNA mixture was denatured at 95°C for 3 min and then cooled to room temperature in stepwise by 50°C for 15s, 30°C for 15s, and 20°C for 10 min before it was denatured at 95°C for 3 min and then cooled to room temperature in stepwise by 50°C for 15s, 30°C for 15s, and 20°C for 10 min before it was placed on ice. (2) The first-step solution was added with 8.5 μ L of reaction mixture containing primers at a concentration of 0.5 μ mol/L each, 0.4 mg/mL bovine serum albumin, 2 mmol/L of dNTP, 10U of the Phi29 DNA polymerase, and 1 μ L reaction buffer. The reaction was carried out at 30°C for 16h and terminated at 65°C for 10 min. Using the RCA products as template, HBV cccDNA was further amplified and quantified with TaqMan real-time PCR

mediated by a pair of cccDNA-selective primers and a probe that targets the gap region between the two direct repeat regions (DR1 and DR2) of the HBV genome. Our cccDNA quantification technique is based on Yanwei Zhong's procedure published in 2011 [5].

All plasma and liver tissue samples were collected at the Military Hospital 103 and the Institute of Biomedicine and Pharmacology, Vietnam Military Medical University, Vietnam.

* *Statistical analysis:* HBV pgRNA and cccDNA levels were measured in log₁₀ copies/mL and log₁₀ copies/cell, respectively, for data analysis. Statistical analyses were performed using MedCalc version 20.019 (MedCalc Software Ltd, Ostend, Belgium).

3. Ethics

The data for this article is based on a portion of the data from a research project titled "Development of a Quantitative Procedure and Assessment of the Fluctuation of Plasma HBV-RNA Load in Response to Treatment in Chronic Hepatitis B Patients in Hanoi"; project number 01C-0808-2017-3, funded by the Hanoi Department of Science and Technology. The project was evaluated as "PASS" by the Ethics Committee for Biomedical Research of the Vietnam Military

Medical University (according to evaluated as "Excellent" at the city Decision No. 780/QD-VMMU dated level according to Decision No. March 28, 2018). The project was 349/QD SKHCN, dated June 23, 2020.

RESULTS

Table 1. Characteristics of participants.

Characteristics	Total (n = 105)	HBeAg (-) (n = 46)	HBeAg (+) (n = 59)	p
Age, years	38.48 ± 12.65	44.74 ± 13.13	33.61 ± 9.89	< 0.001*
Gender (M/F)	95/10	39/7	56/3	0.08**
ALT(IU/L) ≥ 200 (n,%)	59 (56.2)	22 (47.8)	37 (62.7)	0.13*
HAI score ≥ 9 (n,%)	57 (54.3)	29 (50.9)	28 (49.1)	0.11**
Fibro ≥ 3 (n,%)	52 (49.5)	16 (30.8)	36 (69.2)	0,007**

Studen T-test*, *Chi-square test*

The distribution of gender, ALT level, and HAI score did not show significant differences between the HBeAg-positive and HBeAg-negative groups (all $p > 0.05$). However, there were significant differences observed in age and fibrosis level ($p < 0.05$) between the two groups. The male proportion was higher than that of females in both groups.

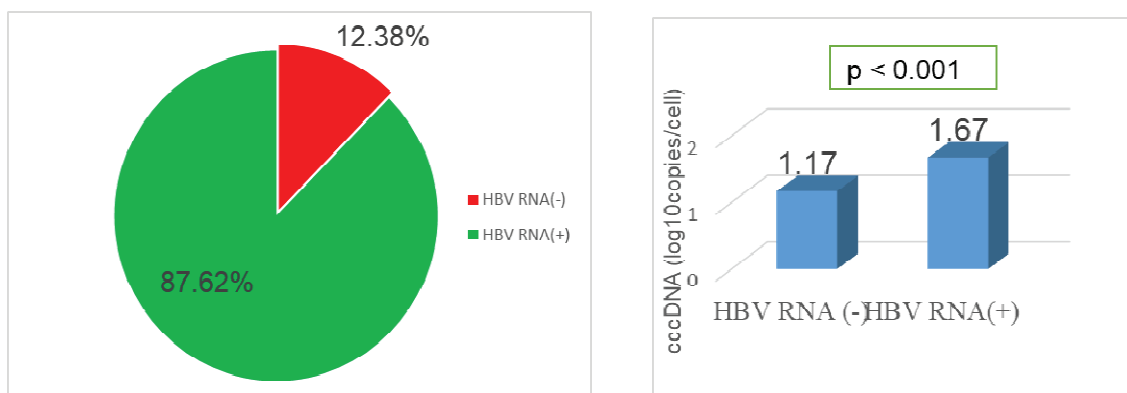


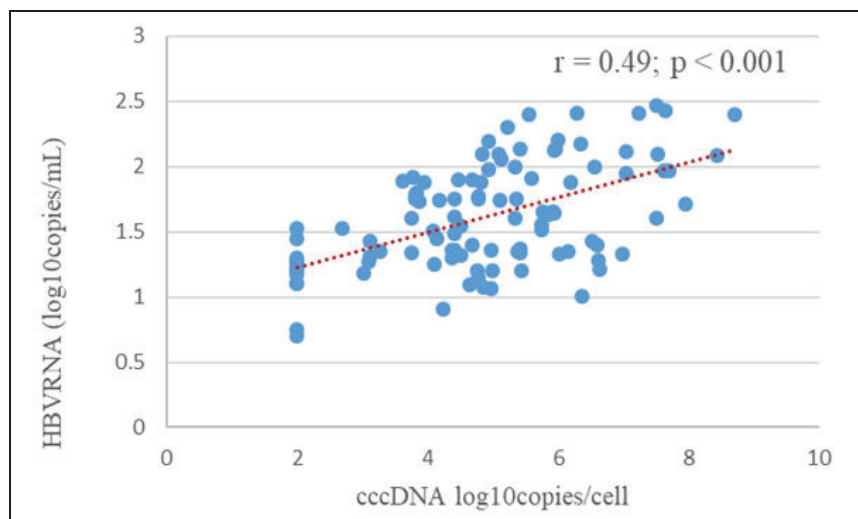
Figure 1. Characteristics of serum HBV RNA and intrahepatic cccDNA levels according to serum HBV RNA levels in the participants.

Regarding serum HBV pgRNA quantification, 87.6% of patients tested positive for HBV pgRNA, while 12.38% tested negative, indicating the prevalence of detectable HBV pgRNA in the study cohort. cccDNA intrahepatic in the group of HBV RNA positive patients group was higher than the negative group; the difference was statistically significant with $p < 0.001$.

Table 2. Intrahepatic cccDNA and serum HBV RNA in participants.

	Total (n = 105)	HBeAg (-) (n = 46)	HBeAg (+) (n = 59)	p
cccDNA (log ₁₀ copies/cell)	1.61 ± 0.40	1.54 ± 0.35	1.67 ± 0.43	0.1
HBV RNA (log ₁₀ copies/mL)	4.88 ± 1.65	4.19 ± 1.45	5.42 ± 1.56	< 0.001

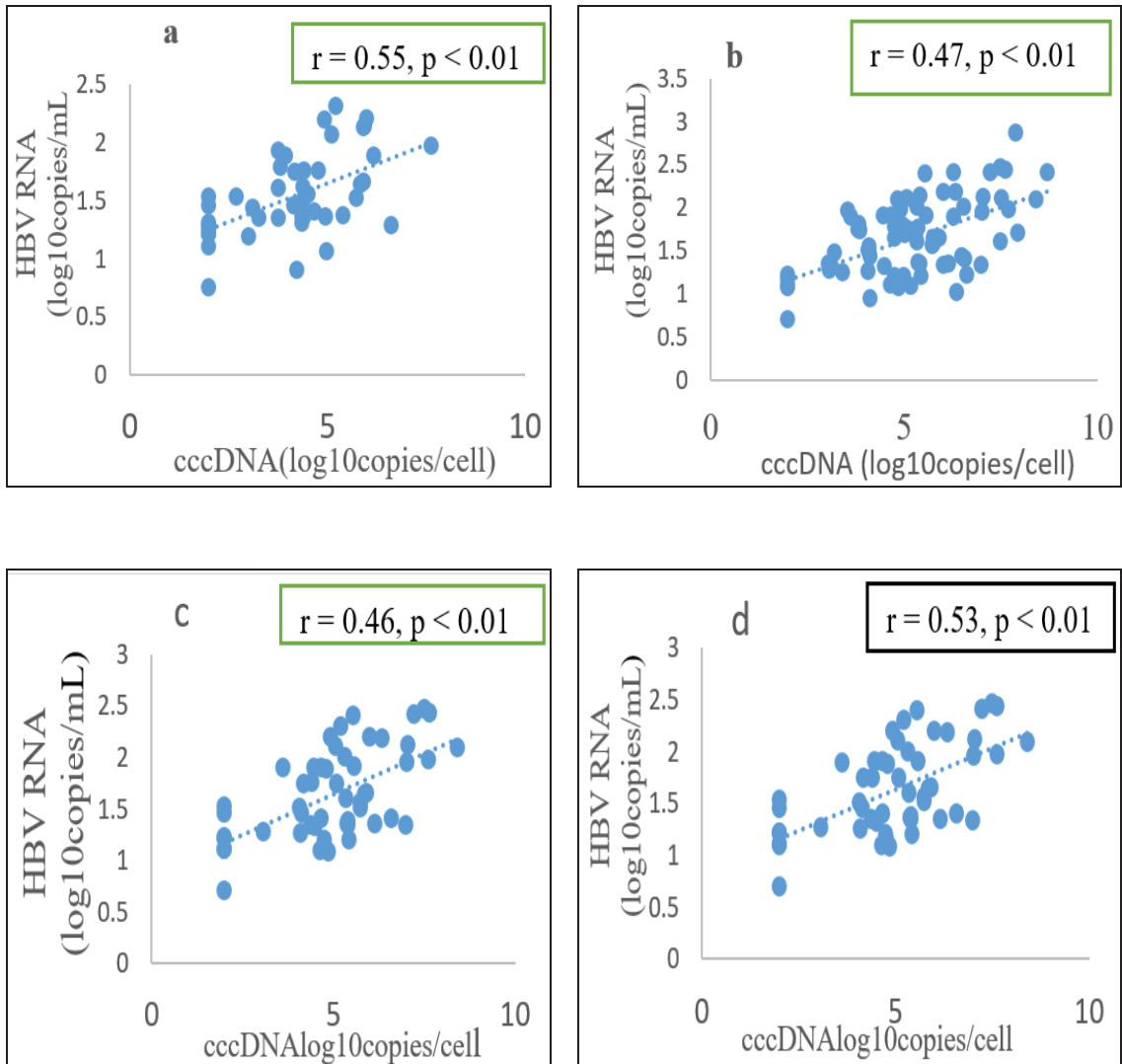
The mean level of HBV pgRNA was measured at 4.88 ± 1.65 log₁₀ copies/mL. The mean level of cccDNA was 1.61 ± 0.4 log₁₀ copies/cell in the total group. cccDNA intrahepatic in the group of HBeAg positive and negative was similar, HBV RNA was higher than HBeAg positive group than the negative group; the difference was statistically significant with $p < 0.001$.



Solid line: The linear growth trend; r: The correlation coefficient.

Figure 2. Correlation between intrahepatic cccDNA and plasma HBV pgRNA in CHB patients.

When assessing the relationship between hepatocyte cccDNA and plasma HBV pgRNA, we found a moderate correlation with a correlation coefficient of $r = 0.49$ ($p < 0.001$).

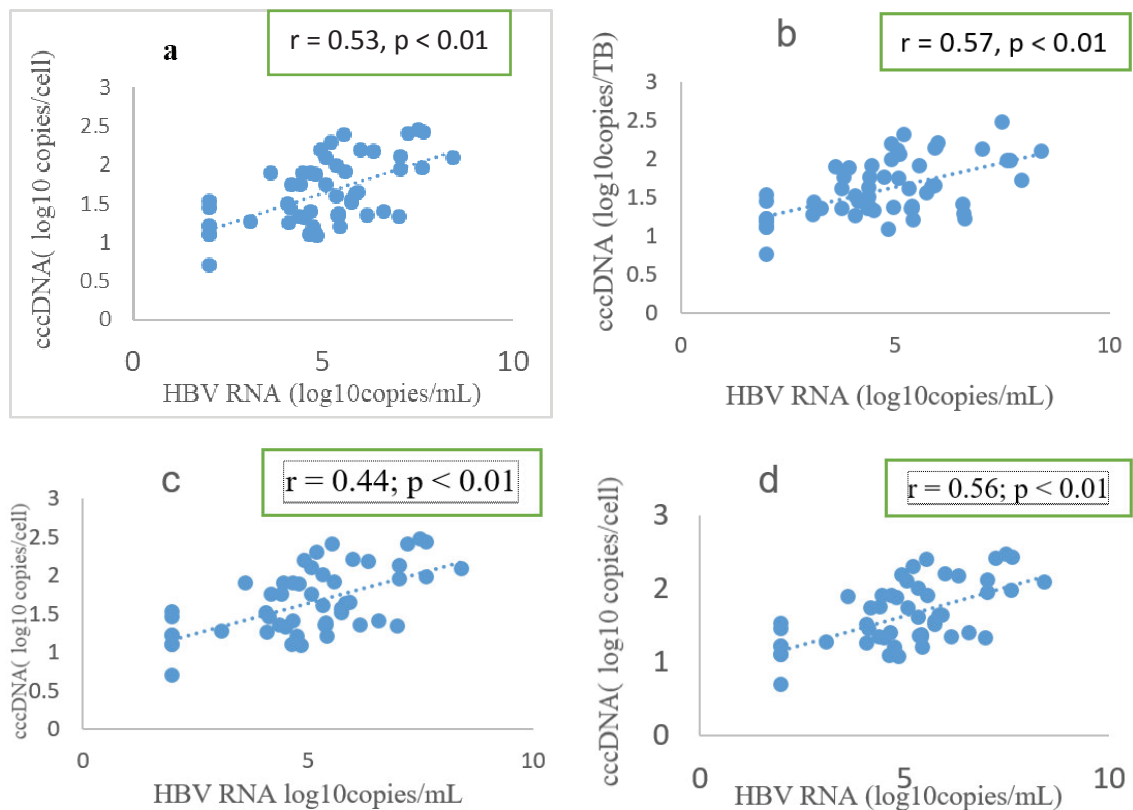


Spearman

(a): HBeAg-negative group ($n = 46$); (b): HBeAg-positive group ($n = 59$);
 (c): ALT < 200 U/L(46); (d): ALT \geq 200 U/L($n = 59$).

Solid line: The linear growth trend; r : the correlation coefficient.

Figure 3. Subgroup analysis of the correlation between cccDNA and HBV pgRNA in CHB patients.



(a): HAI score < 9 (n = 48); (b): HAI score ≥ 9 (n = 57);
 (c): Fibro < 3 (n = 53); (d): Fibro ≥ 3 (n = 52).

Figure 4. Subgroup analysis of the correlation between cccDNA and HBV pgRNA in CHB patients.

DISCUSSION

The mean level of HBV pgRNA was measured at 4.88 ± 1.65 log₁₀ copies/mL. Comparing our results with previous studies, we observed variations in the quantification of plasma HBV pgRNA levels among different patient cohorts. Florian van Bommel's study on 62 patients with HBV reported a mean HBV pgRNA level of 5.2 ± 1.6 log₁₀ IU/mL (equivalent to 5.9 ± 2.3 log₁₀ copies/mL)

[6]. Florian van Bommel's (2015) study also found the results in HBV RNA concentrations between two groups of patients with CHB- HBeAg (+) and CHB- HBeAg (-): 4.9 ± 1.3 log₁₀ IU/mL (5.6 ± 2.0 log₁₀ copies/mL) compared to 4.3 ± 1.4 log₁₀ IU/mL (5.0 ± 2.1 log₁₀copies/mL). In our study, a higher proportion of patients exhibited quantifiable HBV pgRNA in plasma, while the concentration of HBV pgRNA was lower compared to

Yi-Wen Huang's study [7]. These discrepancies may be attributed to differences in the assay design, including the use of specific primer regions targeting different genomic sequences. Additionally, variations in patient demographics, such as geography and ethnicity, between our study and previous investigations could account for the observed differences.

Our study measured the concentration of cccDNA in liver tissue and found it to be $1.61 \pm 0.4 \log_{10}$ copies/cell (*Table 2*), which was higher than the concentration reported by author Xiameo Wang in 2021 (30 and 34 copies/cell equal 1.47 and $1.53 \log_{10}$ copies/cell) [8]. In the subgroup analysis, the cccDNA concentration in the CHB HBeAg positive group was $1.67 \pm 0.43 \log_{10}$ copies/cell, slightly higher than the $1.54 \pm 0.35 \log_{10}$ copies/cell in the HBeAg negative group (*Table 2*); however, the difference was not statistically significant ($p > 0.05$). Our findings aligned with Liang's research, which also showed higher intrahepatic cccDNA levels in HBeAg positive patients compared to HBeAg negative patients, possibly due to increased viral protein transcription and secretion triggering the host immune response [9]. The comparison of intrahepatic cccDNA levels among different studies was crucial for understanding the

dynamics of viral replication in CHB patients. Our study revealed a higher cccDNA concentration in liver tissue compared to a previous study by author Xiameo Wang. This discrepancy could be attributed to variations in sample populations, laboratory techniques, or other experimental factors. Additionally, in the subgroup analysis based on HBeAg status, we observed a trend of slightly higher cccDNA levels in HBeAg positive patients; however, the difference was not statistically significant. This suggests that HBeAg status alone may not be the sole determinant of cccDNA concentration in liver tissue.

Further subgroup analysis based on HBeAg, ALT, HAI, and Fibro levels showed positive correlations between cccDNA and HBV pgRNA. Specifically, the HBeAg-negative group exhibited a higher correlation coefficient with cccDNA compared to the HBeAg-positive group ($r = 0.55$; $p < 0.01$ and $r = 0.47$; $p < 0.01$) (*Figure 3a, 3b*). The ALT group with values ≥ 200 U/L also showed a stronger correlation coefficient than the ALT group with values < 200 U/L ($r = 0.53$; $p < 0.001$ compared with $r = 0.46$; $p < 0.01$). (*Figure 3c, 3d*). Moreover, in the CHB subgroup with HAI scores ≥ 9 points and severe liver fibrosis (fibro ≥ 3), we observed a strong positive correlation

between hepatocyte cccDNA and serum HBV pgRNA compared to the group with less liver damage and mild cirrhosis (HAI ≥ 9 and < 9 points: $r = 0.57$ and 0.53 ; $p < 0.01$) (Fibro ≥ 3 and < 3 points: $r = 0.56, 0.44$; $p < 0.01$).

Comparing our results with those of other studies, Yuhua Gao's research on "HBV DNA, RNA, and HBsAg: which marker has a better correlation with cccDNA before and after treatment with NA?" found a weak correlation between hepatocellular cccDNA levels and plasma HBV pgRNA ($r = 0.25$, $p = 0.02$) in HBeAg-positive CHB patients [10]. In contrast, our study showed a stronger correlation between cccDNA and HBV pgRNA in the HBeAg-positive CHB group, with a correlation coefficient of $r = 0.47$ ($p < 0.001$). The differences in sample populations and testing procedures for cccDNA and HBV pgRNA among various studies may account for these dissimilar results. Likewise, the research conducted by authors Wang and Xiumei Chi (2021) on HBeAg-positive CHB patients before treatment also showed a strong positive correlation between cccDNA and HBV pgRNA ($r = 0.78$, $p < 0.001$) [8]. The determination of plasma HBV pgRNA levels in untreated CHB patients and its association with intrahepatic cccDNA were essential to understand the

dynamics of viral replication, its potential implications, and its potential for disease management. Further research is warranted to explore the clinical significance of plasma HBV pgRNA levels and their utility in guiding treatment decisions for patients with CHB. Our findings open the way for future investigations that may improve the clinical management of CHB and contribute to the development of more personalized therapeutics.

CONCLUSION

Our study demonstrates a significant correlation between intrahepatic cccDNA and plasma HBV pgRNA levels in treatment-naïve CHB patients. The cccDNA levels were higher in the HBV pgRNA-positive group compared to the negative group. The findings suggest that HBV pgRNA may serve as a potential marker for predicting cccDNA activity in CHB patients. However, further investigations and validation of these associations are needed to better understand their clinical implications in HBV infection management.

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