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Original Article

Serum PreS1 antigen combined with vascular endothelial growth factor can identify low-level hepatitis B virus DNA replication in patients with hepatitis B e antigen-negative chronic infection

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Abstract: Background: Patients with hepatitis B e antigen (HBeAg)-negative chronic hepatitis B virus (HBV) infection exhibiting low-level viremia (HBV DNA 20-2000 IU/mL) represent a clinical "gray zone" with uncertain disease activity. Simple serologic markers are needed when high-sensitivity HBV DNA testing is unavailable. Objective: To evaluate serum PreS1 antigen combined with vascular endothelial growth factor (VEGF) for identifying low-level HBV DNA replication in HBeAg-negative chronic HBV infection. Methods: This single-center retrospective study enrolled 178 patients with HBeAg-negative chronic HBV infection (Xi'an No.1 Hospital, January-June 2025). Based on HBV DNA quantification, patients were classified into suppressed (< 20 IU/mL, n = 118) and low-level HBV DNA replication (20-2000 IU/mL, n = 60) groups. Serum PreS1 antigen (by enzyme-linked immunosorbent assay [ELISA]), and VEGF (by quantitative ELISA) were measured. Logistic regression and receiver operating characteristic (ROC) curves were used. Results: The low-level HBV DNA replication group had higher PreS1 positivity (53.3% vs. 4.2%, P < 0.001) and higher VEGF levels (191.75 ± 64.15 vs. 123.36 ± 39.74 pg/mL, P < 0.001). PreS1 positivity (adjusted odds ratio [OR] = 37.209) and each one-standard-deviation increase in VEGF (adjusted OR = 4.596) were independent risk factors. Area under the ROC curve (AUC) for PreS1 alone was 0.745 (sensitivity 53.3%, specificity 95.8%); for VEGF alone, 0.812 (sensitivity 61.7%, specificity 91.5% at 172.32 pg/mL cutoff). The combined model achieved AUC 0.900 (sensitivity 80.0%, specificity 93.2% at probability cutoff 0.328). Conclusions: Serum PreS1 antigen combined with VEGF showed excellent diagnostic performance for identifying low-level HBV DNA replication in HBeAg-negative chronic HBV infection, offering a practical adjunct to high-sensitivity HBV DNA testing.

Keywords: Hepatitis B virus, PreS1 antigen, vascular endothelial growth factor, low viremia, hepatitis B e antigen-negative, diagnostic performance

Introduction

Chronic hepatitis B virus (HBV) infection remains a significant public health issue both in China and globally. The World Health Organization estimates that there are approximately 296 million chronic HBV carriers worldwide, with about 820,000 dying each year from liver failure, liver cirrhosis, and hepatocellular carcinoma (HCC) related to HBV infection [1]. China is a region with a moderately high prevalence of HBV infection. Although the widespread vaccination of hepatitis B vaccines has significantly reduced the incidence of new infections in recent years, the large number of chronic HBV patients still face long-term disease management burdens [2, 3]. Nucleos(t)ide analogue antiviral therapy has enabled effective suppression of HBV DNA in many chronic hepatitis B (CHB) patients. However, how to accurately identify the patient groups that need to initiate antiviral treatment and how to monitor the risk of disease progression during long-term follow-up have always been core issues in clinical practice [4, 5].

In the traditional classification, the hepatitis B e antigen (HBeAg)-negative serological pattern

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(hepatitis B surface antigen [HBsAg]-positive, antibody to hepatitis B e antigen [anti-HBe]-positive, and antibody to hepatitis B core antigen [anti-HBc]-positive) is common. However, this pattern includes two distinct groups with different prognoses. One group comprises inactive HBsAg carriers [6-8]. Their HBV DNA is < 2000 IU/mL or undetectable, alanine aminotransferase (ALT) levels are normal, and liver histology shows no significant inflammation or fibrosis. They have a favorable prognosis and usually do not require antiviral treatment. The other group consists of patients with HBeAg-negative CHB. They have high HBV DNA (> 2000 IU/mL) and/or elevated ALT, with varying degrees of liver inflammation and fibrosis. They have an increased risk of disease progression and need active antiviral therapy [9, 10]. In recent years, domestic and international guidelines have continuously broadened and simplified the indications for CHB treatment. The 2024 World Health Organization guidelines proposed expanding and simplifying the indications for CHB treatment. The 2025 American Association for the Study of Liver Diseases/Infectious Diseases Society of America guidelines and the 2025 European Association for the Study of the Liver guidelines further included "gray zone" patients (i.e., those in the transitional period between active and non-active immunity, not meeting the traditional treatment criteria) in the scope of individualized treatment assessment. However, in clinical practice, some patients with the "HBeAg-negative chronic HBV infection" state have HBV DNA levels ranging from 20 to 2000 IU/mL, with normal or only slightly fluctuating ALT levels [11]. This "gray zone" population is not truly non-active. Studies have confirmed that even HBeAg-negative patients with HBV DNA < 2000 IU/mL may still have significant liver inflammation, necrosis, and fibrosis progression risks, and even a higher risk of HCC [12-14]. Therefore, how to accurately identify these potential active disease patients using simple and economical serologic markers under limited resources has important clinical significance [15].

Currently, highly sensitive HBV DNA quantitative testing is the gold standard for determining the level of viral replication, but it has high costs and long testing time, and its accessibility is limited in some grassroots medical institutions [16, 17]. The routine liver function marker ALT is relatively simple and easy to obtain, but it is insufficiently sensitive in identifying low-level viral replication - a considerable proportion of patients with low-level HBV DNA replication still have ALT levels within the normal range [18]. The quantitative level of HBsAg has predictive value, but its specificity is limited. Therefore, finding supplementary serologic markers that can reflect the viral replication activity and the degree of liver pathologic damage is key to optimizing the stratified management of "HBeAg-negative chronic HBV infection" patients [19].

PreS1 antigen is an important structural component located on the surface of HBV Dane particles [20]. Its coding region overlaps with the viral polymerase gene and directly participates in the binding process between the virus and the surface receptors of liver cells. Studies have confirmed that the presence of serum PreS1 antigen is closely related to the level of HBV DNA ($r = 0.616$), and this correlation is stronger than that between traditional HBeAg and HBV DNA ($r = 0.391$). Especially in HBeAg-negative CHB patients, PreS1 antigen positivity is highly consistent with HBV DNA positivity, with a diagnostic sensitivity of up to 85.8% and a specificity of 93.6% for detecting HBV replication and infection. Therefore, PreS1 antigen is considered to be a more sensitive and more directly reflective serological marker for active viral replication and infection compared to HBeAg [21]. More importantly, PreS1 antigen detection is simple to operate and has a low cost. It can be completed using the enzyme-linked immunosorbent assay (ELISA) method and is suitable for application in all levels of medical institutions.

Vascular endothelial growth factor (VEGF) is a key factor in angiogenesis and the regulation of vascular permeability [22]. During the natural course of CHB, continuous viral replication can induce chronic inflammatory responses in the liver and the formation of hypoxic microenvironments, thereby activating the hypoxia-inducible factor (HIF-1α) signaling pathway and upregulating VEGF expression. Studies have shown that as CHB progresses to liver fibrosis and cirrhosis, the expression levels of VEGF in liver tissues and serum gradually increase, and it is positively correlated with the degree of fibrosis. Moreover, VEGF can also serve as a serological indicator for differentiating the degree of liver fibrosis in patients with chronic hepatitis and cirrhosis, with a diagnostic sensitivity of up to 90% and a specificity of 78% [23, 24]. This sug-

gests that the serum VEGF level not only reflects the hepatic inflammatory activity but also may serve as a non-invasive alternative marker for evaluating the progression of liver fibrosis.

Combining the direct marker reflecting the viral replication status (PreS1 antigen) with the indirect marker reflecting the pathological and physiological damage of the host liver (VEGF) may provide a more comprehensive assessment of "HBeAg-negative chronic HBV infection" patients from the dual dimensions of "virus-host interaction" [25]. Theoretically, even when HBV DNA is at a low level of replication, the persistent viral packaging and release (manifested as positive PreS1 antigen) can still drive the inflammatory response and pathological angiogenesis (manifested as elevated VEGF levels) in the liver microenvironment. The combined detection of these two markers is expected to more sensitively and specifically identify "HBeAg-negative chronic HBV infection" patients with low-level HBV DNA replication but with potential risk of disease progression, thereby providing a reference basis for clinical antiviral treatment decisions.

Based on the above background, this study used a retrospective case-control research method, including 178 patients with "HBeAg-negative chronic HBV infection" who visited Xi'an No.1 Hospital from January to June 2025. Using high-sensitivity HBV DNA detection as the gold standard for grouping, the study aimed to evaluate the recognition efficacy of serum PreS1 antigen combined with VEGF detection for low-level HBV DNA replication, with the expectation of providing a simple and efficient new strategy for optimizing the individualized stratified management of "HBeAg-negative chronic HBV infection" patients.

Materials and methods

Research design and ethical statement

This study was a single-center, retrospective case-control study. The research protocol was approved by the Ethics Committee of Xi'an No. 1 Hospital. As it is a retrospective analysis of clinical data, the Ethics Committee waived the requirement for written informed consent from patients. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

Research subjects

Clinical data and serological test results were continuously collected from patients with chronic HBV infection (referred to as "HBeAg-negative chronic HBV infection") who visited the outpatient department or were hospitalized in the Infection Department/Liver Disease Department of Xi'an No.1 Hospital from January to June 2025.

Inclusion criteria: (I) Age ≥ 18 years; (II) Serum HBsAg positive for more than 6 months, with HBeAg negative, anti-HBe positive, and anti-HBc positive (i.e., "HBeAg-negative chronic HBV infection" serological pattern); (III) Have complete liver function biochemical tests, quantitative HBV serological markers, high-sensitivity HBV DNA quantitative, serum PreS1 antigen, and VEGF test results within the same period (within 1 week).

Exclusion criteria: (I) Current infection with hepatitis A virus, hepatitis C virus (HCV), hepatitis D virus, hepatitis E virus, or human immunodeficiency virus (HIV); (II) Complicated with alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), autoimmune liver disease, drug-induced liver injury, or genetic metabolic liver disease; (III) Previously or currently receiving any form of anti-HBV treatment (including nucleos(t)ide analogues and interferons); (IV) Complicated by decompensated liver cirrhosis, HCC or other malignant tumors; (V) Complicated by severe heart, lung, kidney or blood system diseases; (VI) Pregnant or lactating women; (VII) Within the past 3 months, a history of major trauma, surgery, or blood transfusion; (VIII) Patients with incomplete clinical data.

A total of 386 consecutive patients with HBeAg-negative chronic HBV infection were initially screened at Xi'an No.1 Hospital from January to June 2025. After sequential application of the exclusion criteria (**Figure 1**), 208 patients were excluded: 18 with co-infection (hepatitis A virus, HCV, hepatitis D virus, hepatitis E virus, or HIV), 26 with other liver diseases (alcoholic, NAFLD, autoimmune, drug-induced, or metabolic), 54 with prior or current anti-HBV therapy, 14 with decompensated cirrhosis, HCC, or other malignancy, 16 with severe cardiac/pulmonary/renal/hematological diseases, 4 pregnant or lactating, 11 with recent major trauma/surgery/blood transfusion, and 65 with incomplete clinical or laboratory data (missing HBV

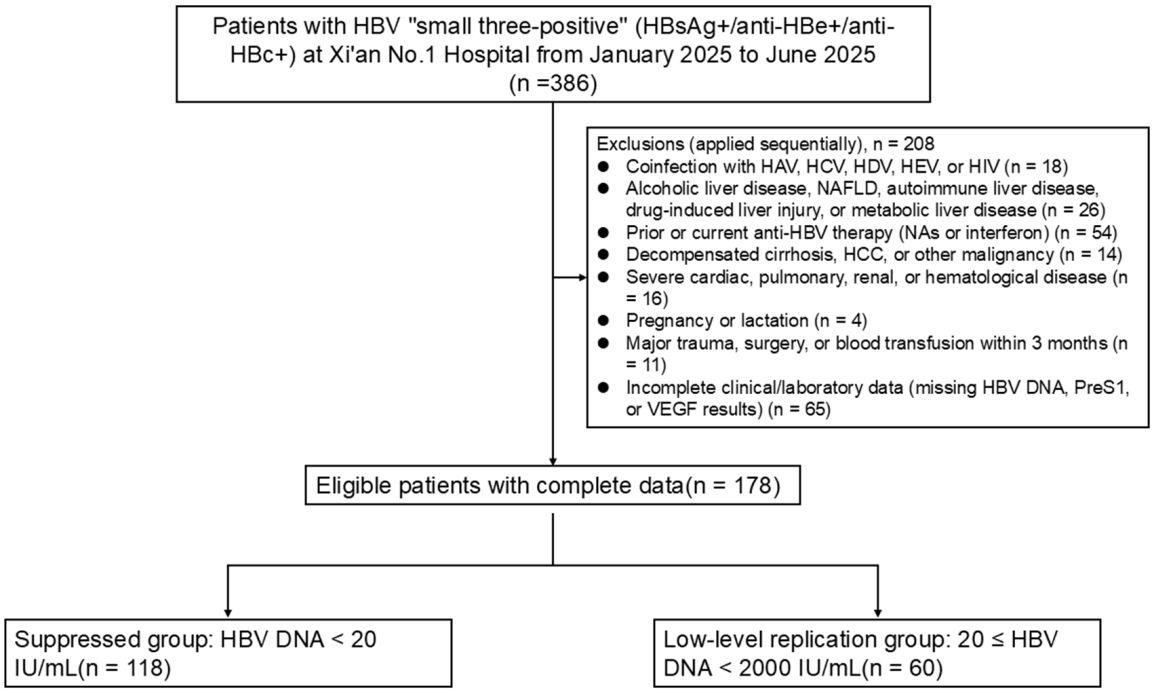

Figure 1. Flow chart of patient enrollment. Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; anti-HBe, antibody to hepatitis B e antigen; anti-HBc, antibody to hepatitis B core antigen; HAV, hepatitis A virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; NAFLD, non-alcoholic fatty liver disease; NAs, nucleos(t)ide analogues; HCC, hepatocellular carcinoma; PreS1, pre-S1 antigen of hepatitis B virus; VEGF, vascular endothelial growth factor.

DNA, PreS1 antigen, or VEGF results). The remaining 178 patients had complete data for all key variables (PreS1 antigen, VEGF, HBV DNA, ALT, HBsAg, and liver function panel) and were included in the final analysis. Therefore, no data imputation was required.

In addition, 25 healthy control subjects (HBsAg-negative, normal liver function) and 25 patients with non-HBV chronic liver disease (NAFLD or ALD, HBsAg-negative) were enrolled to assess marker specificity. Their serum PreS1 antigen and VEGF levels were measured using the same methods as described above. Detailed data are presented in Table S1. Healthy controls were recruited from the health checkup center during the same study period. They had no history of liver disease, normal liver function tests (ALT ≤ 40 U/L, aspartate aminotransferase [AST] ≤ 40 U/L, gamma-glutamyl transferase [GGT] ≤ 60 U/L), and negative HBsAg, anti-HCV, and anti-HIV. Non-HBV chronic liver disease patients were selected from the outpatient hepatology clinic, diagnosed with NAFLD (n = 15) or ALD (n = 10) based on standard criteria, and were HBsAg-negative with no evidence of HBV infection. Written informed consent was obtained from all control subjects. The inclusion of control groups was approved by the Ethics Committee of Xi'an No.1 Hospital.

Group definition

Based on the results of highly sensitive HBV DNA testing and the current clinical guidelines thresholds, the enrolled patients were divided into two groups: (1) Suppressed group: HBV DNA < 20 IU/mL (i.e., below the detection limit). (2) Low-level viremia group: 20 IU/mL ≤ HBV DNA < 2000 IU/mL (**Figure 1**).

Data collection and detection methods

All patients' clinical data and laboratory test results were retrospectively extracted from the hospital laboratory information system and electronic medical record system. General clinical data included the patient's age, gender, height, and weight, and the body mass index (BMI = weight (kg)/height^2 (m^2)) was calculated.

The liver function biochemical indicators were detected using an automatic biochemical ana-

lyzer (Beckman Coulter AU5800, USA). The specific items included serum ALT (normal reference value: 9-50 U/L for males, 7-40 U/L for females), AST (normal reference value: 15-40 U/L for males, 13-35 U/L for females), GGT (normal reference value: 10-60 U/L for males, 7-45 U/L for females), alkaline phosphatase (normal reference value: 45-125 U/L), total bilirubin (normal reference value: 3.4-20.5 μmol/L), direct bilirubin (normal reference value: 0-6.8 μmol/L), albumin (ALB, normal reference value: 35-55 g/L), and total protein (normal reference value: 60-80 g/L). Globulin was calculated by subtracting ALB from total protein, and the normal reference value was 20-40 g/L.

The quantitative detection of HBV serological markers was performed using the chemiluminescent microparticle immunoassay. The Abbott ARCHITECT i2000SR automatic immunoanalyzer and the original factory-recommended reagent kits (Abbott Diagnostics, USA) were used. The test items included quantitative determination of serum HBsAg (detection range: 0.05-250 IU/mL; after sample dilution, it could reach 125,000 IU/mL), antibody to hepatitis B surface antigen (normal reference value: < 10 mIU/mL considered negative, ≥ 10 mIU/mL positive), HBeAg (normal reference value: sample absorbance/cutoff value [S/CO] < 1.0 negative), anti-HBe (normal reference value: S/CO > 1.0 negative, ≤ 1.0 positive), and anti-HBc (normal reference value: S/CO < 1.0 negative).

The HBV DNA quantification test was conducted using a highly sensitive real-time quantitative polymerase chain reaction method. The Roche cobas 6800 automatic molecular diagnostic system and the corresponding reagent kit (Roche Diagnostics, Germany) were employed. The detection limit was 20 IU/mL, and the linear range was 20-1.7 × 10^8 IU/mL. Nucleic acid extraction, amplification, and fluorescence signal detection were carried out strictly in accordance with the instrument's standard operating procedures. Negative controls, positive controls, and internal quality controls were set for each test.

The serum PreS1 antigen detection was performed using the double-antibody sandwich ELISA. The reagent kit was purchased from Shanghai Kehua Biotechnology Co., Ltd. and the operation was carried out strictly in accordance with the instructions. The result interpretation criteria were as follows: an S/CO ratio ≥ 1.0 was judged as positive, and < 1.0 was negative. Negative controls, positive controls, and blank controls were set for each batch of tests.

The serum VEGF concentration was measured by ELISA. The kit was purchased from R&D Systems (USA), with a detection range of 31.2-2000 pg/mL and a minimum detection limit of 9.0 pg/mL. All samples were tested in the same batch, and the addition, incubation, washing, and color development were carried out strictly in accordance with the instructions. The absorbance was measured using a microplate reader (Thermo Scientific Multiskan FC, USA) at a wavelength of 450 nm. The sample VEGF concentration was calculated based on the standard curve. The intra-assay coefficient of variation was < 8%, and the inter-assay coefficient of variation was < 12%.

The non-invasive liver fibrosis score was calculated based on serological indicators. AST-to-platelet ratio index (APRI) was calculated as: [(AST/upper limit of normal)/platelet count (× 10^9/L)] × 100. The normal upper limit of AST (upper limit of normal) was set at 40 U/L. An APRI value < 0.5 indicated no obvious liver fibrosis, an APRI value ≥ 1.5 suggested significant liver fibrosis, and an APRI value > 2.0 indicated a high possibility of cirrhosis. Fibrosis-4 index (FIB-4) was calculated as: [age (years) × AST (U/L)]/[platelet count (× 10^9/L) × sqrt(ALT (U/L))]; a FIB-4 value < 1.45 indicated no obvious liver fibrosis, and a FIB-4 value ≥ 3.25 suggested significant liver fibrosis. The platelet count data were derived from the concomitant blood routine test and were measured using an automatic blood analyzer (Sysmex XN-9000, Japan).

Quality control

To ensure data accuracy, all laboratory tests were conducted in accordance with standard operating procedures at the Laboratory Department of Xi'an No.1 Hospital. The testing instruments were regularly calibrated, and the reagents were used within their valid periods. The extraction of clinical data was independently completed and cross-checked by two researchers, and if there was any disagreement, a third researcher made the final decision. During the data analysis stage, all codes

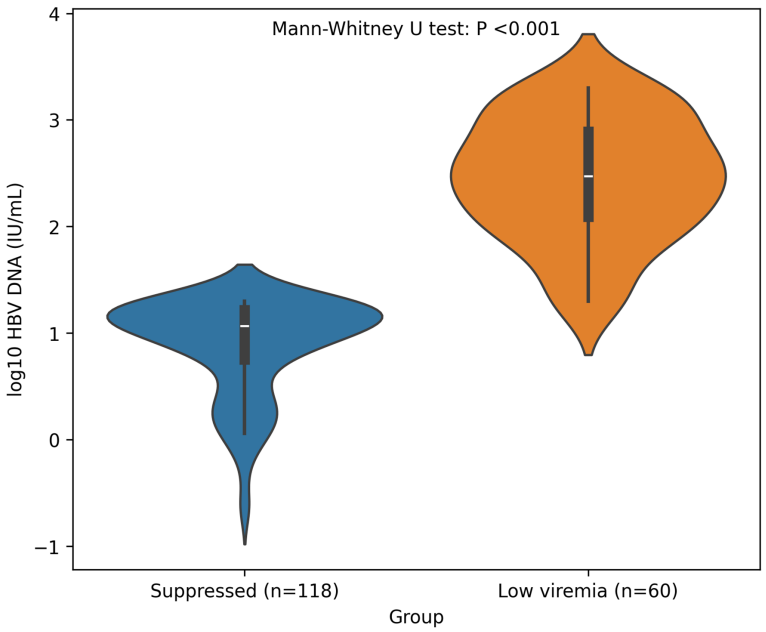

Figure 2. Violin plot of HBV DNA levels in the two groups. Data are presented as $\log_{10}$-transformed HBV DNA (IU/mL). The violin plot shows the kernel density distribution; the inner box represents the IQR with the median indicated by a white dot. Groups: suppressed group (HBV DNA < 20 IU/mL, n = 118) and low-level replication group (20 ≤ HBV DNA < 2000 IU/mL, n = 60). The *P*-value from the Mann-Whitney U test is shown (P < 0.001). The horizontal dashed line indicates the lower limit of the low-level viremia group (20 IU/mL, log10(20) ≈ 1.30). Abbreviations: HBV, hepatitis B virus; IU, international unit; IQR, interquartile range.

and results were reviewed by statistical professionals.

Statistical analysis

All statistical analyses were performed using SPSS 26.0 and Python 3.10. A two-tailed P < 0.05 was considered significant.

Continuous variables were expressed as mean ± standard deviation or median (interquartile range) depending on the results of normality tests (Shapiro-Wilk test). Between-group comparisons used the independent-samples t-test, Mann-Whitney U test, or chi-square test, as appropriate.

Univariate and multivariate binary logistic regression were used to identify factors associated with low-level HBV DNA replication. Variables with P < 0.10 by univariate analysis or those deemed clinically important (age, gender) were entered into a forward stepwise multivariate model. Adjusted odds ratios (OR) with 95% confidence intervals (CI) were reported. Multicollinearity was assessed by variance inflation factor; variance inflation factor > 5 indicated significant multicollinearity (Table S2).

Handling of missing data: As described in Section 2.2 and **Figure 1**, the final 178 patients had complete key data; therefore, no imputation was performed. The intention-to-treat principle was not applicable; a complete-case (per-protocol) analysis was conducted.

Diagnostic performance of PreS1 antigen, VEGF, and their combination was evaluated by receiver operating characteristic curves. The combined probability was derived from logistic regression: Logit(P) = $\beta_0 + \beta_1 \times$ PreS1-Ag + $\beta_2 \times \log_{10}$(VEGF), with coefficients provided in the Results section. Areas under the curve (AUCs) were compared using DeLong's test. Youden's index determined optimal cutoffs.

Correlation analysis used the Pearson or Spearman correlation coefficient. All figures were generated with GraphPad Prism 9.0 and Python Matplotlib.

Results

Patient baseline characteristics

A total of 178 patients with HBeAg-negative chronic HBV infection were included: 118 in the suppressed group (HBV DNA < 20 IU/mL) and 60 in the low-level viremia group (20-2000 IU/mL). The two groups were well balanced in age, gender, and BMI (all P > 0.05).

Compared to the suppressed group, the low-level viremia group had significantly higher levels of ALT, AST, GGT, alkaline phosphatase, total bilirubin, HBsAg, PreS1 positivity, and VEGF, as well as lower platelet counts and elevated APRI/FIB-4 scores (all P < 0.001). ALB levels did not differ significantly (P = 0.181). HBV DNA levels were completely separated by design (**Figure 2**). These findings indicated that even with low-level viremia, patients exhibited

Table 1. Baseline characteristics of the two groups

Variable	Suppressed (n = 118)	Low-level viremia (n = 60)	$t/\chi^2/U$	*P*-value
Age	43.40 ± 10.89	43.40 ± 10.29	-0.001	0.999
Gender	55 (46.6%)	32 (53.3%)	0.476	0.490
BMI	24.27 ± 3.64	24.23 ± 3.90	0.057	0.955
ALT	18.84 (11.10-31.44)	48.32 (27.73-63.18)	1332.000	< 0.001
AST	16.05 (8.79-26.61)	47.38 (26.39-65.94)	1148.000	< 0.001
GGT	25.88 (14.13-36.86)	44.58 (24.26-68.62)	2050.500	< 0.001
ALP	57.87 (38.49-87.33)	89.27 (56.09-130.16)	2318.000	< 0.001
TBIL	9.45 (4.24-14.82)	18.35 (12.72-26.94)	1661.500	< 0.001
ALB	42.21 ± 4.16	41.41 ± 3.56	1.343	0.181
PLT	242.90 ± 42.86	208.21 ± 38.87	5.397	< 0.001
HBsAg_quant	811.55 (267.20-2241.99)	2258.61 (875.05-6571.09)	2211.000	< 0.001
HBV DNA	11.66 (5.64-16.44)	296.55 (124.77-777.57)	0.000	< 0.001
PreS1-Ag	5 (4.2%)	32 (53.3%)	55.284	< 0.001
VEGF	123.36 ± 39.74	191.75 ± 64.15	-7.554	< 0.001
APRI	0.17 (0.09-0.27)	0.55 (0.34-0.79)	926.500	< 0.001
FIB-4	0.65 (0.45-0.95)	1.48 (0.98-1.88)	1135.000	< 0.001

Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; TBIL, total bilirubin; ALB, albumin; PLT, platelet count; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; VEGF, vascular endothelial growth factor; APRI, aspartate aminotransferase-to-platelet ratio index; FIB-4, fibrosis-4 index; IQR, interquartile range; PreS1-Ag, pre-S1 antigen of hepatitis B virus.

significant hepatic inflammatory activity and risk of fibrosis. Detailed baseline data are presented in **Table 1**.

Expression characteristics of PreS1 antigen and VEGF in each group

In terms of PreS1 antigen expression, the positive rate in the suppressed group was only 4.2% (5/118), while the positive rate in the low-level viremia group was as high as 53.3% (32/60), and the difference between the groups was significant ($\chi^2 = 55.284$, P < 0.001, **Figure 3**). This result demonstrated a significant enrichment of PreS1 antigen positivity in the low-level viremia group, indicating that even at HBV DNA levels below 2000 IU/mL, PreS1 antigen can effectively indicate the active packaging and replication status of the virus.

In terms of serum VEGF levels, the VEGF concentration in the low-level viremia group was 191.75 ± 64.15 pg/mL, significantly higher than that in the suppressed group (123.36 ± 39.74 pg/mL) (independent-samples t-test: t = -7.554, P < 0.001, **Figure 4**).

Specificity assessment using control groups

As shown in Table S1, PreS1 antigen was positive in 0% (0/25) of healthy controls and 4.0% (1/25) of non-HBV chronic liver disease patients, compared to 53.3% (32/60) in the low-level viremia group (both P < 0.001). Serum VEGF levels in the low-level viremia group (191.75 ± 64.15 pg/mL) were significantly higher than those in healthy controls (85.43 ± 22.31 pg/mL) and non-HBV chronic liver disease patients (132.61 ± 28.52 pg/mL) (both P < 0.001, independent-samples t-test).

These results confirm the high specificity of PreS1 antigen for HBV replication and demonstrate that VEGF elevation in low-level viremia exceeds that seen in other common liver diseases.

Analysis of factors affecting low-level viral replication

To identify the independent risk factors associated with low-level HBV DNA replication, a univariate logistic regression analysis was conducted. The results are presented in **Table 2**. The analysis revealed that PreS1 antigen positivity was a strong predictor of low-level replication, with an unadjusted OR of 25.829 (95% CI: 9.227-72.301, P < 0.001). For a one-standard-deviation increase in serum VEGF level, the risk of low-level replication increased 5.001-fold (95% CI: 2.916-8.575, P < 0.001).

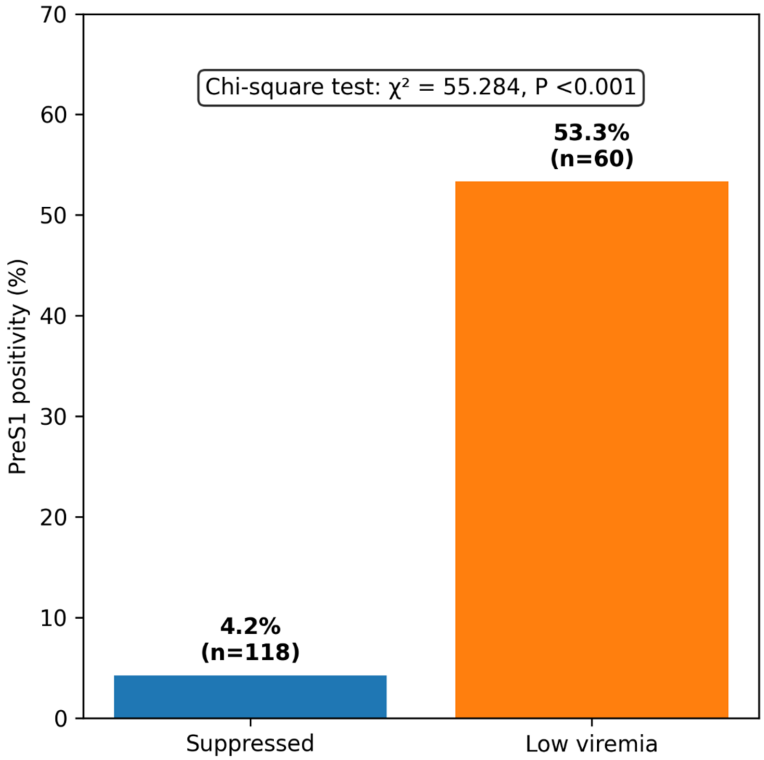

Figure 3. Bar chart comparing PreS1 antigen positivity rates between groups. Bars represent the percentage of patients with positive serum PreS1 antigen. Suppressed group: 4.2% (5/118); low-level replication group: 53.3% (32/60). Numbers above bars indicate exact percentages and sample sizes (n). Error bars are not applicable due to the binary outcome. The chi-square test yielded $\chi^2 = 55.284$, $P < 0.001$. Abbreviations: PreS1, pre-S1 antigen of hepatitis B virus.

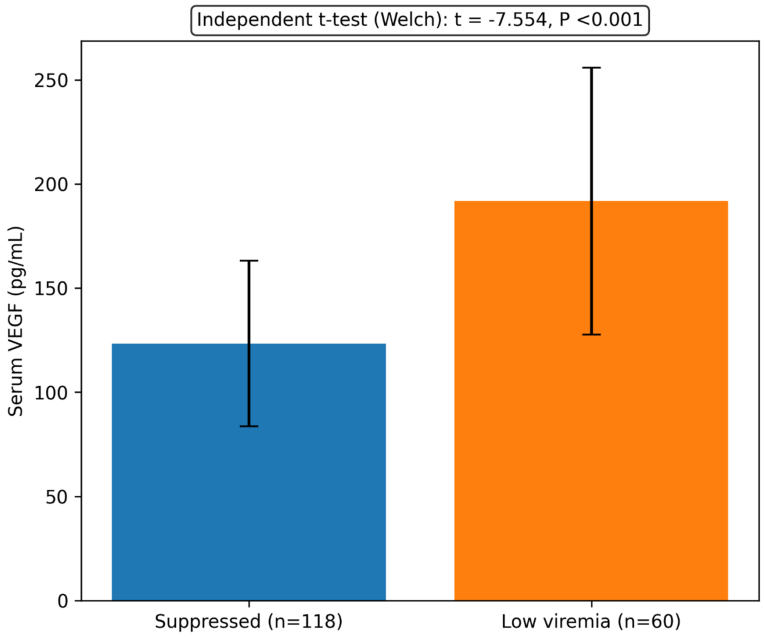

Figure 4. Bar chart of serum VEGF levels in the two groups. Data are presented as mean ± SD. Suppressed group (n = 118): 123.36 ± 39.74 pg/mL; low-level replication group (n = 60): 191.75 ± 64.15 pg/mL. Normality was verified by the Shapiro-Wilk test (suppressed P = 0.682, low viremia P = 0.837). Independent-samples t-test (Welch's correction) showed a significant difference (t = -7.554, P < 0.001). Error bars indicate standard deviation. Abbreviations: VEGF, vascular endothelial growth factor; IQR, interquartile range; SD, standard deviation.

Other variables significantly associated with low-level replication included ALT (OR = 1.059, 95% CI: 1.039-1.079, P < 0.001), AST (OR = 1.065, 95% CI: 1.044-1.086, P < 0.001), $\log_{10}$(HBsAg) (OR = 2.684, 95% CI: 1.658-4.346, P < 0.001), PLT (OR = 0.980, 95% CI: 0.972-0.989, P < 0.001), APRI (OR = 441.086, 95% CI: 67.585-2878.680, P < 0.001), and FIB-4 (OR = 16.455, 95% CI: 6.870-39.412, P < 0.001). Age, gender, and BMI did not show significance in the univariate analysis (all P > 0.05).

The variables with P < 0.10 in the univariate analysis and the clinically important variables (age, gender) were included in the multivariate logistic regression model to correct for potential confounding factors. The results are presented in **Table 3** and **Figure 5**. The multivariate analysis showed that after adjusting for age, gender, ALT, and $\log_{10}$(HBsAg), PreS1 antigen positivity remained an independent risk factor for low-level HBV DNA replication. The adjusted OR was 37.209 (95% CI: 8.633-160.364, P < 0.001). For every one standard deviation increase in serum VEGF, the risk of low-level replication increased 4.596-fold (95% CI: 2.099-10.066, P < 0.001).

Table 2. Univariate logistic regression analysis for low-level HBV DNA replication

Variable	OR	95% CI	*P* value
Age	1.000	0.971-1.030	0.999
Gender	1.309	0.702-2.441	0.397
BMI	0.998	0.918-1.084	0.953
PreS1-Ag	25.829	9.227-72.301	< 0.001
VEGF (per SD)	5.001	2.916-8.575	< 0.001
ALT	1.059	1.039-1.079	< 0.001
AST	1.065	1.044-1.086	< 0.001
Log_{10}(HBsAg)	2.684	1.658-4.346	< 0.001
PLT	0.980	0.972-0.989	< 0.001
APRI	441.086	67.585-2878.680	< 0.001
FIB-4	16.455	6.870-39.412	< 0.001

Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PLT, platelet count; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; VEGF, vascular endothelial growth factor; SD, standard deviation; APRI, aspartate aminotransferase to platelet ratio index; FIB-4, Fibrosis-4 index; PreS1-Ag, pre-S1 antigen of hepatitis B virus; OR, odds ratio; CI, confidence interval.

Table 3. Multivariate logistic regression analysis for low-level HBV DNA replication

Variable	Adjusted OR	95% CI	*P* value
Constant	0.002	0.000-0.045	< 0.001
Age	1.014	0.967-1.063	0.566
Gender	1.334	0.490-3.629	0.572
PreS1-Ag	37.209	8.633-160.364	< 0.001
VEGF (per SD)	4.596	2.099-10.066	< 0.001
ALT	1.020	0.994-1.047	0.128
Log_{10}(HBsAg)	2.703	1.365-5.353	0.004

Abbreviations: ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; VEGF, vascular endothelial growth factor; SD, standard deviation; PreS1-Ag, pre-S1 antigen of hepatitis B virus; OR, odds ratio; CI, confidence interval.

Log_{10}(HBsAg) also retained an independent effect, with an adjusted OR of 2.703 (95% CI: 1.365-5.353, P = 0.004). ALT did not show an independent effect in the multivariate model (adjusted OR = 1.020, 95% CI: 0.994-1.047, P = 0.128), suggesting that the independent predictive value of transaminase levels is limited when both viral replication markers and host response markers are considered. Age and gender also were not significant by multivariate analysis (both P > 0.05).

To further explore the correlations among variables, we conducted a correlation analysis and created a heatmap (**Figure 6**). The results showed that HBV DNA was moderately positively correlated with PreS1 antigen (r = 0.504) and VEGF (r = 0.419). VEGF was strongly correlated with ALT (r = 0.643) and AST (r = 0.642), and also showed positive correlations with APRI (r = 0.616) and FIB-4 (r = 0.488), indicating that VEGF levels may reflect the degree of liver inflammation and fibrosis.

Diagnostic efficacy of each indicator for low-level viral replication

The binary logistic regression model combining PreS1 antigen and log_{10}(VEGF) yielded the equation:

$$Logit(P) = -7.9215 + 2.4855 \times PreS1\text{-}Ag + 3.1081 \times log_{10}(VEGF)$$

where PreS1-Ag = 1 for positive, 0 for negative. The predicted probability is $P = 1/(1 + e^{-Logit(P)})$.

As shown in **Figure 7** and **Table 4**, the AUC for PreS1 antigen alone was 0.745 (sensitivity 53.3%, specificity 95.8%). For VEGF alone, the AUC was 0.812 (sensitivity 61.7%, specificity 91.5% at a cutoff of 172.32 pg/mL). The combined model achieved an AUC of 0.900, higher than that of either single marker alone (DeLong test, both P < 0.05). At the optimal probability cutoff of 0.328, the combined model had a sensitivity of 80.0% and specificity of 93.2%.

Different probability cutoffs can be tailored to clinical needs: a cutoff of 0.2 yielded 95.0% sensitivity and 95.3% negative predictive value, suitable for screening; a cutoff of 0.5 yielded 95.8% specificity (**Table 5**). These results demonstrate that combining PreS1 antigen (a direct marker of viral replication) with VEGF (a marker of host liver damage) significantly improves diagnostic accuracy for low-level HBV DNA replication.

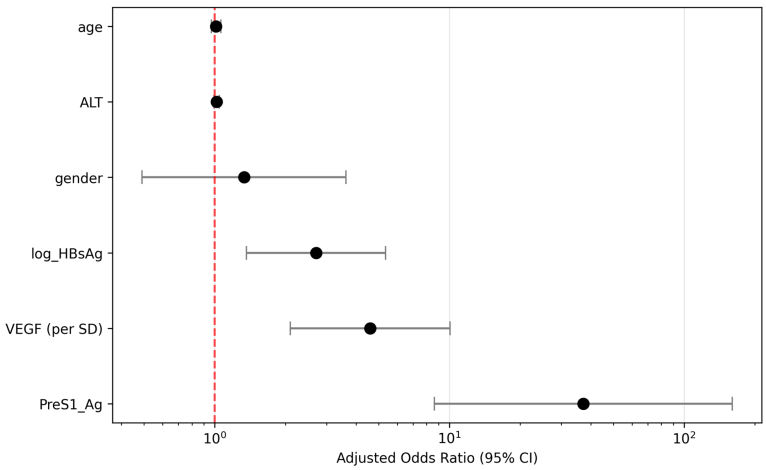

Figure 5. Forest plot of multivariate logistic regression analysis. Adjusted OR with 95% CIs are shown on a logarithmic scale. The model included age (per year), gender (male vs. female), PreS1 antigen (positive vs. negative), VEGF (per SD increase), ALT (per U/L), and $\log_{10}$(HBsAg) (per log10 IU/mL). The vertical dashed line at OR = 1 indicates no association. Variables with OR > 1 and a CI that does not cross 1 are considered independent risk factors. *P* values are given in **Table 3**. Abbreviations: OR, odds ratio; CI, confidence interval; VEGF, vascular endothelial growth factor; ALT, alanine aminotransferase; SD, standard deviation; HBsAg, hepatitis B surface antigen; PreS1-Ag, pre-S1 antigen of hepatitis B virus.

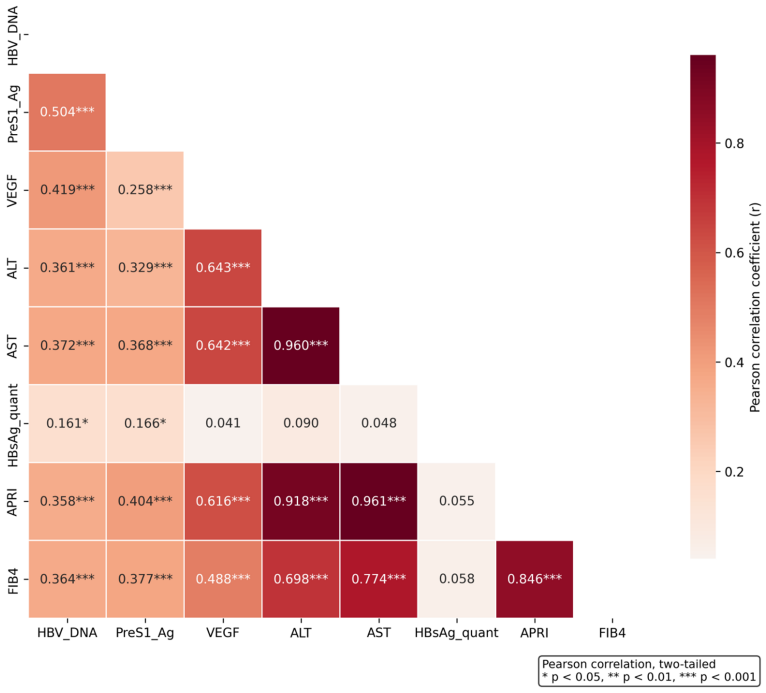

Figure 6. Correlation heatmap of key variables. Pairwise Pearson correlation coefficients (r) are displayed. The color scale ranges from -1 (dark red) to +1 (dark blue), as shown in the color bar. White squares on the upper triangle are masked for clarity. Variables analyzed: HBV DNA (log10), PreS1 antigen (binary, coded as 0/1), VEGF (log10), ALT (log10), AST (log10), HBsAg (log10), APRI, and FIB-4. All correlations with $|r| \geq 0.3$ are considered moderate to strong; notable correlations: PreS1 and HBV DNA (r = 0.504), VEGF and ALT (r = 0.643), VEGF and APRI (r = 0.616). Significance levels are indicated by asterisks (*P < 0.05, ***P < 0.001). All $|r| \geq 0.3$ have P < 0.001. Abbreviations: HBV, hepatitis B virus; VEGF, vascular endothelial growth factor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B surface antigen; APRI, aspartate aminotransferase-to-platelet ratio index; FIB-4, fibrosis-4 index; PreS1-Ag, pre-S1 antigen of hepatitis B virus.

Discussion

This study retrospectively analyzed the clinical and serologic data of 178 patients with "HBeAg-negative chronic HBV infection". This study was the first to evaluate systematically the diagnostic efficacy of serum PreS1 antigen combined with VEGF detection for low-level HBV DNA replication (20-2000 IU/mL). The results showed that in patients with HBeAg-negative chronic HBV infection, the positive rate of PreS1 antigen and serum VEGF level were significantly higher in the low-level viremia group than in the suppressed group. The combination of the two markers achieved an AUC of 0.900, a sensitivity of 80.0%, and a specificity of 93.2%, demonstrating excellent diagnostic efficacy. This finding provides a new serologic strategy for identifying patients in the "gray zone" with potential disease progression risk in clinical practice.

PreS1 antigen is an important component of the HBV envelope, present on the surface of complete Dane particles and tubular particles. Its coding region overlaps with the viral polymerase gene; thus, its expression is closely relat-

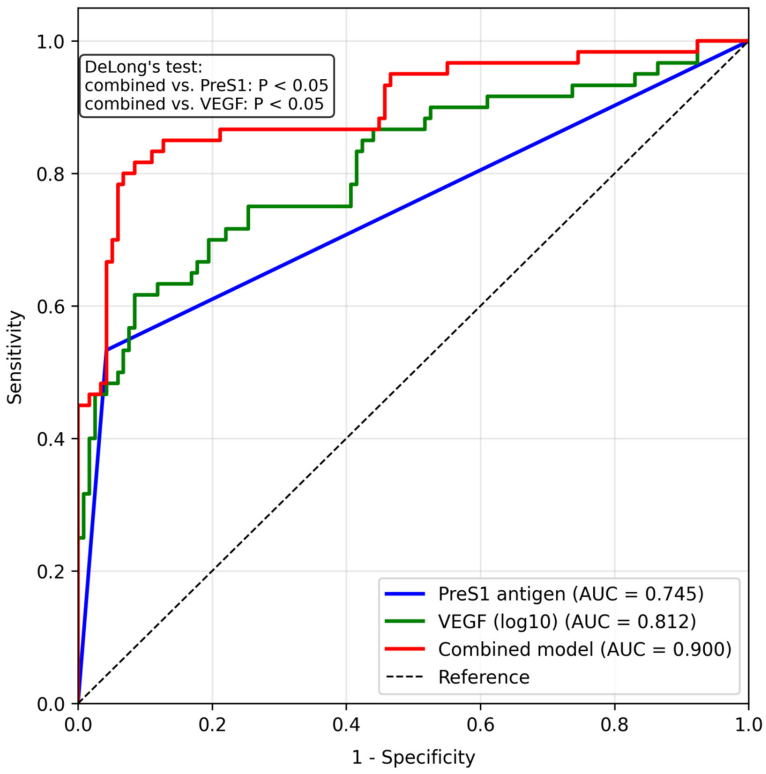

Figure 7. ROC curves for predicting low-level HBV DNA replication. The combined model probability was calculated as: P = 1/(1 + exp(-(-7.9215 + 2.4855 × PreS1-Ag + 3.1081 × $\log_{10}$(VEGF)))), where PreS1-Ag = 1 for positive, 0 for negative, and VEGF is in pg/mL. The AUCs for each marker are as follows: PreS1 antigen alone, 0.745, VEGF ($\log_{10}$) alone, 0.812, combined model, 0.900. Pairwise comparisons of AUCs were performed using the DeLong test: combined vs. PreS1, P < 0.05; combined vs. VEGF, P < 0.05. The diagonal dashed line represents the reference (AUC = 0.5). The optimal cutoff for the combined model was 0.328 (sensitivity 80.0%, specificity 93.2%). Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic; PreS1-Ag, pre-S1 antigen of hepatitis B virus; VEGF, vascular endothelial growth factor.

ed to the viral replication and assembly process [26, 27]. In this study, the positive rate of PreS1 antigen in the low-level viremia group was 53.3%, while it was only 4.2% in the suppressed group (P < 0.001). This significant difference strongly suggests that even within the range of HBV DNA below 2000 IU/mL, PreS1 antigen positivity can effectively distinguish patients with active viral packaging. Notably, there were still 5 cases (4.2%) in the suppressed group that tested positive for PreS1, a phenomenon that is common in clinical practice and may be related to transient low-level viral replication, differences in the sensitivity of the detection method, or variations in the S gene of the virus resulting in low DNA quantification but still expressible envelope proteins [28, 29]. However, the positive rate of this group was significantly lower than that of the low-level viremia group, ensuring the high specificity (95.8%) of PreS1 as a qualitative marker. Consistent with previous studies, the PreS1 antigen was moderately correlated with HBV DNA (r = 0.504), further supporting its feasibility as an alternative marker for viral replication [30].

VEGF is a key factor regulating angiogenesis and vascular permeability. During the chronic HBV infection process, continuous viral replication can induce liver inflammatory responses and hypoxic microenvironments, activating the HIF-1α signaling pathway, thereby upregulating VEGF expression [31, 32]. In this study, the mean serum VEGF concentration in the low-level viremia group was 191.75 ± 64.15 pg/mL, significantly higher than 123.36 ± 39.74 pg/mL in the suppressed group (P < 0.001), indicating that even at the stage of low HBV DNA replication, virus-driven pathologic changes in the liver still exist. Correlation analysis showed that VEGF was strongly correlated with ALT (r = 0.643) and AST (r = 0.642), suggesting that VEGF levels can partially reflect the degree of liver inflammatory injury. Additionally, VEGF was also correlated with APRI (r = 0.616) and FIB-4 (r = 0.488), which was consistent with previous literature showing that VEGF increases with the progression of liver fibrosis [33]. These results collectively indicate that serum VEGF is not only a factor in tumor angiogenesis but can also serve as an indirect serological indicator of liver inflammation and fibrosis progression in the monitoring of CHB.

The mechanistic interplay between PreS1 and VEGF may be mediated through multiple HBV-driven pathways. Persistent viral replication stabilizes HIF-1α, which transcriptionally upregulates VEGF to promote pathologic angiogene-

Table 4. Diagnostic performance of PreS1 antigen, VEGF, and combined model for low-level replication

Marker/Model	AUC	Sensitivity	Specificity	Optimal cutoff
PreS1 antigen	0.745	53.3%	95.8%	Not applicable
VEGF (log10)	0.812	61.7%	91.5%	172.32 pg/mL
Combined model	0.900	80.0%	93.2%	0.328

Abbreviations: AUC, area under the curve; PreS1, pre-S1 antigen of hepatitis B virus; VEGF, vascular endothelial growth factor.

Table 5. Performance of the combined model at different cut-off probabilities

Cutoff probability	Sensitivity	Specificity	PPV	NPV
0.1	98.3%	7.6%	35.1%	90.0%
0.2	95.0%	51.7%	50.0%	95.3%
0.3	81.7%	89.0%	79.0%	90.5%
0.4	61.7%	95.8%	88.1%	83.1%
0.5	53.3%	95.8%	86.5%	80.1%

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

sis and vascular remodeling [34]. Mounting evidence also reveals that HBV-encoded microRNAs such as HBV microRNA-3 repress factor inhibiting hypoxia-inducible factor 1, a negative regulator of HIF-1α, thereby activating the HIF-1α/VEGFA axis [35]. Furthermore, HBV enhances VEGFA expression through N6-methyladenosine-mediated mRNA stabilization via the insulin-like growth factor 2 mRNA-binding protein 3 reader protein, adding an epitranscriptional layer to VEGF regulation [36]. This multi-level virus-host crosstalk provides a mechanistic rationale for the elevated VEGF levels observed in HBeAg-negative patients with low-level HBV replication.

A single biomarker has its limitations in identifying low-level viral replication: the PreS1 antigen, as a qualitative indicator, has extremely high specificity but only 53.3% sensitivity, and it misses nearly half of the patients; VEGF, as a quantitative indicator, has improved sensitivity (61.7%) but is still not ideal. This study constructed a combined prediction model using binary logistic regression, combining the direct biomarker reflecting viral replication status (PreS1) with the indirect biomarker reflecting host liver damage (VEGF), to conduct a comprehensive assessment from the dual dimensions of "virus-host interaction". The AUC of the combined model increased to 0.900, significantly better than the individual application of PreS1 (0.745) or VEGF (0.812) (DeLong test, both $P < 0.05$). At the optimal cutoff probability of 0.328, the sensitivity of the combined detection reached 80.0%, while the specificity remained at 93.2%, effectively compensating for the deficiencies of a single indicator. This result has clear clinical translational value: for patients with HBeAg-negative chronic HBV infection, if PreS1 antigen is positive and VEGF level is elevated, one should be highly vigilant for low-level viral replication and potential liver damage, and we recommend shortening the follow-up interval or further performing a liver biopsy assessment; conversely, if both indicators are negative, the long-term prognosis is relatively favorable.

In the multivariate analysis, ALT was not independently associated with low-level HBV DNA replication after adjusting for PreS1 antigen and VEGF (adjusted OR = 1.020, $P = 0.128$). This confirms that ALT alone had limited predictive value in this setting. Relying solely on ALT levels would miss a considerable proportion of patients with low viremia, as approximately 20-30% of CHB patients with normal ALT may still have significant liver injury [27]. By contrast, the combined detection of PreS1 and VEGF effectively overcame this limitation, providing more accurate identification of at-risk individuals, as reflected by the superior AUC of the dual-marker model (0.900 vs. 0.745 for PreS1 alone and 0.812 for VEGF alone).

This study has the following limitations. First, as a single-center retrospective study, there is inherent selection bias, and the conclusions need to be verified by multicenter, prospective cohort studies. Second, the sample size is relatively limited (n = 178), especially the low-level viremia group has only 60 cases, which may affect the statistical power of some subgroup analyses. Third, there is no liver biopsy pathology examination as a gold standard control, and we could not directly evaluate the correlation between the combined model of PreS1 and

VEGF and liver inflammation grading and fibrosis staging. Fourth, the PreS1 antigen detection used an ELISA qualitative method. If quantitative detection by chemiluminescence were adopted, it would further improve diagnostic sensitivity. Fifth, to partially address this limitation, we have included small control groups of healthy individuals and non-HBV chronic liver disease patients (Table S1), which confirm the high specificity of PreS1 antigen and the marked elevation of VEGF in the low-level viremia group compared to non-HBV liver diseases. Nevertheless, larger-scale studies are needed to definitively establish disease specificity. Sixth, VEGF levels are affected by various factors (such as platelet count and degree of tissue hypoxia). Although this study excluded patients with other systemic diseases, there may still be unmeasured confounding factors.

Future research could explore the following aspects: conducting prospective cohort studies using liver biopsy pathology or FibroScan as the endpoint to verify the predictive value of PreS1 combined with VEGF for significant liver fibrosis (≥ F2); exploring quantitative detection technology for PreS1 antigen to establish a more refined risk stratification model; evaluating the applicability of the combined model across different ALT levels and age subgroups; and combining other non-invasive fibrosis indicators (such as FIB-4 and APRI) to construct a multi-dimensional comprehensive scoring system that further improves the accuracy of prognosis assessment for patients with low-level replication.

Conclusion

The combined detection of serum PreS1 antigen and VEGF had excellent diagnostic efficacy for low-level HBV DNA replication in patients with HBeAg-negative chronic HBV infection (AUC = 0.900, sensitivity 80.0%, specificity 93.2%). PreS1 antigen positivity was an independent risk factor for low-level replication, and elevated serum VEGF levels were also closely associated with this condition. The combination of these two markers provides a comprehensive assessment across two dimensions: viral replication activity and host liver damage. It could serve as an economical and convenient supplementary method to high-sensitivity HBV DNA testing, providing new evidence for optimizing individualized stratification management of patients with HBeAg-negative chronic HBV infection and guiding antiviral treatment.

Disclosure of conflict of interest

None.

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Table S1. Comparison of PreS1 antigen positivity and serum VEGF levels among healthy controls, non-HBV chronic liver disease patients, and the two study groups

Group	n	PreS1 positive, n (%)	VEGF (pg/mL), mean ± SD
Healthy controls	25	0 (0.0%)	85.43 ± 22.31
Non-HBV chronic liver disease*	25	1 (4.0%)	132.61 ± 28.52
Suppressed group (HBV DNA < 20 IU/mL)	118	5 (4.2%)	123.36 ± 39.74
Low-level viremia group (20-2000 IU/mL)	60	32 (53.3%)	191.75 ± 64.15

*Non-HBV chronic liver disease includes patients with NAFLD or ALD, all HBsAg-negative. Abbreviations: PreS1, pre-S1 antigen of hepatitis B virus; HBV, hepatitis B virus; VEGF, vascular endothelial growth factor; SD, standard deviation; IQR, interquartile range; NAFLD, non-alcoholic fatty liver disease; ALD, alcoholic liver disease; CLD, chronic liver disease; HBsAg, hepatitis B surface antigen. PreS1 positivity: healthy vs. low-level viremia group, $P < 0.001$ (chi-square test); non-HBV CLD vs. low-level viremia group, $P < 0.001$. VEGF levels: healthy vs. low-level viremia group, $P < 0.001$ (independent-samples t-test); non-HBV CLD vs. low-level viremia group, $P < 0.001$ (independent-samples t-test).

Table S2. VIF analysis for the final multivariate logistic regression model

Variable	VIF	VIF < 5 (Yes/No)
Age	1.01	Yes
Gender	1.05	Yes
PreS1-Ag	1.17	Yes
VEGF (per SD)	1.55	Yes
ALT	1.65	Yes
Log_{10}(HBsAg)	1.06	Yes

All VIF values < 5 indicate no significant multicollinearity. Abbreviations: VIF, variance inflation factor; PreS1-Ag, pre-S1 antigen of hepatitis B virus; VEGF, vascular endothelial growth factor; SD, standard deviation; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen.