Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

The Significance of HBV-miR-3 in Predicting the Efficacy of Nucleoside Analogue Therapy in Patients with Chronic Hepatitis B

Zhi-Hao Zeng
Central South University
Yao Yang
Central South University
Zhen-Yu Xu
Central South University
Hai-Zhen Zhu
Central South University
Zhong-Shang Dai
Central South University
Min Zhang
zhangmin001@csu.edu.cn

Central South University

Article

Keywords: Hepatitis B virus, HBV-miR-3, Predictive significance, NA therapy, Performance

Posted Date: March 10th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-7819645/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

BACKGROUND

Hepatitis B virus (HBV) is a major global health threat, with chronic infection affecting 300 million people and causing 800,000 annual deaths from complications like cirrhosis and hepatocellular carcinoma, while nucleoside analogs (NAs)—key antiviral therapies—cannot eliminate covalently closed circular DNA (cccDNA) and require long-term use with varying safety profiles. HBV-encoded microRNA-3 (HBV-miR-3), a functionally diverse viral miRNA linked to viral replication, immune evasion, and carcinogenesis with predictive value for pegylated interferon-α efficacy, has an unexplored role in evaluating NA therapy.

AIM

To investigate the predictive significance and advantages of HBV-miR-3 levels for NA therapeutic efficacy through a randomized clinical trial of patients receiving long-term NA therapy.

METHODS

This registered randomized trial (NCT03903796) included 53 adults with chronic HBV infection (HBsAg-positive ≥6 months, HBV DNA ≥2×10⁴ IU/mL) without concurrent infections or advanced liver disease. Patients were randomized 2:1 to TMF (25 mg/day) or TDF (300 mg/day) for 48 weeks, followed by TMF monotherapy up to week 144. Serum HBV-miR-3, HBV DNA, HBV RNA, HBsAg, and HBeAg were quantified using real-time PCR at baseline and follow-ups (weeks 12, 24, 48, 72, 96, 144). Statistical analyses included t-tests, ANOVA, correlation analyses, logistic regression, and receiver operating characteristic (ROC) curves to assess predictive performance.

RESULTS

Baseline characteristics were balanced between TMF and TDF groups. At week 48, patients with undetectable HBV DNA (DNA-negative group) had significantly lower baseline HBV-miR-3, HBV DNA, HBV RNA, and HBeAg levels, and were older, compared to the DNA-positive group. Over 144 weeks, HBV-miR-3, HBV DNA, HBV RNA, HBsAg, and HBeAg declined more markedly in the DNA-negative group. HBV-miR-3 showed strong correlations with HBV DNA, HBV RNA, and HBeAg during early treatment (≤24 weeks). ROC analyses revealed HBV-miR-3 at baseline, week 12, and week 24 independently predicted week-48 HBV DNA negativity (positive predictive values [PPV] 74.5%, 73.3%, 77.1%, respectively), with performance comparable to HBV RNA and superior to HBsAg. Notably, combining HBV-miR-3 with HBV DNA yielded higher PPVs (up to 100% at week 12).

CONCLUSION

HBV-miR-3 is closely associated with HBV replication and serves as a robust predictor of HBV DNA virological response during NA therapy.

Core Tip

HBV-miR-3, a viral microRNA linked to HBV replication and immune evasion, demonstrated strong predictive value for nucleoside analog (NA) efficacy in a randomized trial (NCT03903796). Baseline and early-treatment (≤24 weeks) HBV-miR-3 levels independently predicted week-48 virological response (HBV DNA negativity; PPVs 74.5–77.1%), correlating with HBV DNA/RNA/HBeAg decline. Combined with HBV DNA, its predictive performance improved (PPV up to 100%), highlighting its dual role as a dynamic biomarker and potential therapeutic target in chronic HBV infection.

INTRODUCTION

Hepatitis B Virus (HBV) is a small hepatotropic, non-cytopathic DNA virus that infects humans, and constitutes one of the major global health threats[1]. HBV infection is highly prevalent across all regions worldwide, with approximately one-third of the global population having been exposed to HBV and around 300 million people living with chronic infection. Complications arising from HBV infection, such as liver fibrosis, cirrhosis, and hepatocellular carcinoma, result in approximately 800,000 deaths annually[2]. This imposes a substantial public health burden, making antiviral therapy a critical intervention to ameliorate the current situation.

Currently, the widely used anti-HBV agents include pegylated interferon-alpha (PegIFN-α) and nucleoside analogs (NAs). NAs primarily target the polymerase region of HBV to interfere with viral replication; however, they cannot completely eliminate the covalently closed circular DNA (cccDNA) of HBV, necessitating long-term or even lifelong administration to mitigate the risk of recurrence[3]. In China, four NAs have been approved for clinical use: entecavir (ETV), tenofovir disoproxil fumarate (TDF), tenofovir alafenamide (TAF), and tenofovir amibufenamide (TMF) [4]. Among these, TDF exhibits an extremely low resistance rate and favorable long-term efficacy, maintaining a high response rate even in patients with ETV resistance. Nevertheless, it carries risks of nephrotoxicity and osteopenia, particularly in elderly patients or those in menopause[5]. TAF and TMF have demonstrated higher bioactivity and biochemical response rates, with improved safety profiles for renal and bone function, but are associated with a higher incidence of dyslipidemia[6,7]. Thus, evaluating the efficacy of NA therapy in patients with chronic hepatitis B remains a clinically significant issue of widespread concern.

HBV possesses a small, relaxed circular DNA (rcDNA) genome of only 3.2 kb, which is packaged within an icosahedral nucleocapsid composed primarily of 240 copies of core protein subunits[8]. Due to the unique structure of cccDNA, HBV produces various unconventional RNAs during transcription, such as pregenomic RNA (pgRNA) variants formed by redundant fragments and microRNAs (miRNAs) [9,10]. MiRNAs are a class of endogenous non-coding single-stranded RNA molecules, approximately 22 nucleotides in length. They regulate gene expression at the post-transcriptional level by complementary pairing with target mRNAs, and are involved in multiple biological processes including cell proliferation, differentiation, and apoptosis[11]. HBV encodes five distinct miRNAs, among which HBV-encoded microRNA-3 (HBV-miR-3) has been identified as the one with the most diverse biological functions and

the highest abundance. Studies have shown that HBV-miR-3 is highly expressed in HBV-related hepatocellular carcinoma (HCC) tissues or HBV-integrated HCC cells[12]. Furthermore, HBV-miR-3 has been confirmed to participate in HBV replication, expression, immune evasion, and the initiation and progression of HCC. Its specific mechanism may involve targeting mRNAs of the S and C regions in the viral genome, thereby affecting the assembly and release of viral particles by downregulating protein expression levels[13]. Additional research indicates that HBV-miR-3 can interfere with the cGAS-Sting-IFN signaling pathway, playing a role in HBV immune evasion[14]. In patients with chronic HBV infection, HBV-miR-3 has also been shown to contribute to HCC development by targeting molecules such as PTEN and FIH-1[15]. In HBV-infected patients, HBV-miR-3 levels exhibit a significant positive correlation with HBV DNA and HBsAg[12]. Thus, HBV-miR-3 holds potential as a marker for evaluating therapeutic efficacy during the treatment of patients with chronic HBV infection.

Our preliminary studies have demonstrated that HBV-miR-3 has a strong predictive value for the efficacy of PegIFN-α therapy in HBeAg-positive patients[16]. However, to date, no studies have investigated the role of HBV-miR-3 in evaluating the efficacy of NA therapy, and the exploration of its target sites and related signaling pathways in cccDNA replication and expression remains largely underexplored. Therefore, this study aims to explore the predictive significance and advantages of HBV-miR-3 levels for NA efficacy through a randomized clinical trial of patients receiving long-term NA therapy.

MATERIALS AND METHODS

Ethical approval

This study is a randomized clinical trial registered on http://clinicaltrials.gov, with the registration number NCT03903796. The first registration was on 16/08/2018. This study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University (No. 2022001) and was conducted in accordance with the Declaration of Helsinki. We got informed consent from all study participants.

Study population

Patients included in this study fulfilled all the following inclusion criteria: 1. All patients were aged over 18 years, with female participants being non-pregnant and non-lactating. 2. Serologically confirmed persistent HBsAg positivity for more than 6 months. 3. Either HBeAg-positive or HBeAg-negative, with HBV DNA levels ≥2×10^4 IU/mL. 4. No exposure to interferon therapy within 6 months prior to the baseline assessment. Patients were excluded if they met any of the following conditions: 1. Concurrent infection with HCV, HIV, or HDV. 2. Radiologically confirmed hepatocellular carcinoma. 3. Clinical evidence of hepatic decompensation, including ascites, hepatic encephalopathy, or variceal bleeding. 4. A history of solid organ or bone marrow transplantation. 5. Comorbid severe renal, cardiovascular, pulmonary, or neurological disorders.

Study design

In this study, participants were randomly assigned to the TMF treatment group or the TDF treatment group at a ratio of 2:1. During the first 48 weeks, the TMF group received TMF (HS-10234) at a dose of 25 mg once daily, while the TDF group received TDF (GlaxoSmithKline, Tianjin, China) at 300 mg once daily. Concomitantly, the TMF group was administered a TDF placebo at 300 mg once daily, and the TDF group received a TMF placebo at 25 mg once daily. From week 49 to week 144, all enrolled patients were switched to TMF monotherapy at 25 mg once daily. Based on HBV DNA levels measured at the week 48 follow-up, patients were categorized into a DNA-positive group and a DNA-negative group.

All enrolled patients completed follow-up visits at weeks 12, 24, 48, 72, 96, and 144 after the baseline visit (week 0). At each follow-up, basic vital signs including body weight, pulse, blood pressure, and electrocardiogram were recorded. Approximately 20 ml of peripheral blood was collected for general biochemical tests (complete blood count, liver function, renal function, electrolytes, and blood lipids) as well as virological assays. Additionally, liver elastography was performed to dynamically assess the presence of progressive cirrhosis in patients.

Quantification of HBV-miR-3 and HBV-RNA

Serum microRNAs were extracted using TRIzol reagent: 100 μL serum was mixed with 500 μL TRIzol and 2.5 μL synthetic C.elegans miR-39 (25 pmol/L, internal control), incubated at room temperature for 10 min, then mixed with 200 μL chloroform. After centrifugation (4°C, 12,000×g, 15 min), 300 μL of the upper aqueous phase was collected, combined with 300 μL isopropanol, and processed using a small RNA adsorption column with sequential washes (washing solution 1 once, washing solution 2 twice) followed by elution with 15 μL RNase-free water. Reverse transcription was performed by mixing 10 μL extracted microRNA with 5 μL 3× reverse transcription mix, incubated at 42°C for 30 min and 70°C for 5 min to generate cDNA. Duplex quantitative real-time PCR was conducted in 50 μL reactions containing 35 μL PCR Mix and 15 μL cDNA. Amplification conditions were: 95°C for 3 min, followed by 50 cycles of 95°C for 12 s and 60°C for 30 s (fluorescence detection). HBV-miR-3 and internal control were detected via FAM and HEX channels, respectively. Ct values and quantitation were determined using standard curves generated from 4 serial dilutions of HBV-miR-3 standards (6.0×10^3–6.0×10^7 copies/mL) (Primer names and sequences:

HBV-miR3-RT primer: GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAGAAGAAC. HBV-miR3-PCR forward primer: TGCGGATCTTCTTGTTGGTT. HBV-miR3probe: FAM-CACTGGATACGACAAACGCCGCAGA-BHO1) .

HBV RNA was extracted from 200μL serum using a CiZhu- based nucleic acid extraction kit according to the manufacturer's instructions: samples (including negative/positive controls) were mixed with lysis buffer, incubated with binding buffer at 60°C for 10 min, and processed via magnetic separation. After sequential washes with buffer A and B, RNA was eluted in 30 μL elution buffer using RNase-free materials to avoid contamination. Reverse transcription was performed by incubating 10 μL eluted RNA with reverse transcription reagents (including reverse transcriptase, buffer, dNTPs) and the specific anchor primer (5'-ACCACGCTACTCGCTAC-3') at 50°C for 30 min, followed by enzyme inactivation at

70°C for 5 min. Quantitative real-time PCR was conducted in a 7500 instrument with cDNA, using specific primers, fluorescent probes, DNA polymerase, buffer, and dNTPs. Amplification conditions: 95°C for 2 min, followed by 50 cycles of 95°C for 15 s and 60°C for 30 s (annealing/extension), then cooling to 25°C for 10 s. HBV RNA concentrations were calculated automatically via standard curves generated from control Ct values.

Statistical analysis

Data in this study were imported into Python 3.8 for statistical analysis. Continuous variables are presented as mean ± standard error (X ± SE); t-tests were used for comparisons of normally distributed continuous variables, and one-way analysis of variance (ANOVA) was applied for comparisons among multiple groups. Categorical variables are expressed as frequencies (percentages), with intergroup comparisons performed using the chi-square test. The scipy.stats module was utilized for t-tests, one-way ANOVA, and chi-square tests. The seaborn module was employed to generate curves for visualizing dynamic changes and trends of each indicator. Pearson correlation coefficients (R values) were calculated using the scipy.stats module to assess correlations between indicators, and scatter plots with regression lines were generated via the seaborn module to visualize correlations between different markers and miR3. Univariate and multivariate logistic regression analyses were performed to determine odds ratios (OR), 95% confidence intervals (95% CI), and P values, where OR values indicate the correlation between a factor and outcome determination. Receiver operating characteristic (ROC) curves were plotted and area under the curve (AUC) values were calculated; the optimal cutoff value was derived from ROC curves, with positive predictive values (PPV) and negative predictive values (NPV) subsequently computed. A two-tailed P value < 0.05 was considered statistically significant, with a significance level of $\alpha = 0.05$.

RESULTS

The flowchart of this study is shown in Figure 1. Among the 53 enrolled patients, the mean age was 36.89 years, with 67.9% being male. No significant statistical differences in baseline demographic data, blood routine parameters, liver function, virological markers, or HBV-miR-3 levels were observed between patients receiving TDF and TMF treatment within the first 48 weeks (Table 1). Patients were divided into DNA-negative and DNA-positive groups at week 48. No significant differences were found between the two groups in terms of gender, biochemical indicators, or hepatitis B surface antigen levels. However, patients in the DNA-negative group at week 48 were significantly older than those in the DNA-positive group (39.53±1.72 vs 32.16±2.28, p=0.013). Notably, baseline levels of HBV-RNA (4.14±0.39 vs 5.93±0.36, p=0.000), HBV-DNA (6.81±0.17 vs 7.20±0.28, p=0.002), HBV-miR-3 (5.38±0.29 vs 6.54±0.29, p=0.002), and HBeAg (2.14±0.36 vs 0.33±0.32, p=0.001) in the week 48 DNA-negative group were significantly lower than those in the week 48 DNA-positive group (Table 2).

We analyzed the changes in virological indicators over treatment duration in each patient group receiving NA therapy and plotted dynamic curves. Among these, five indicators (HBV-miR-3, HBV DNA, HBV RNA,

HBsAg, and HBeAg) showed substantial declines over the 144-week treatment course, with more pronounced reductions observed in the DNA-negative group. HBsAg levels in the DNA-positive group increased notably during early treatment (within 24 weeks) and late treatment (96–144 weeks), whereas the DNA-negative group showed no such upward trends and maintained a consistent downward trajectory throughout the entire treatment period (Figure 2). Subsequently, we performed correlation analyses between HBV-miR-3 levels and other virological indicators at weeks 0, 12, 24, and 48 of treatment. During early treatment (within 24 weeks), HBV DNA, HBV RNA, and HBeAg exhibited strong correlations with HBV-miR-3 levels, with the strongest correlation observed for HBV DNA ($R = 0.595$, 0.552, and 0.517, respectively; $p = 0.0001$). At week 48, HBV RNA ($R = 0.329$, $p = 0.038$), HBeAg ($R = 0.408$, $p = 0.004$), and HBsAg ($R = 0.308$, $p = 0.035$) showed moderate correlations with HBV-miR-3 levels, while the correlation between HBV DNA and HBV-miR-3 at week 48 was weaker (Figure 3). Over the 144-week follow-up, a significant correlation was observed between HBV-miR-3 negativity and HBV DNA negativity ($p = 0.049$) (Table 3). Collectively, these findings suggest that reductions in viral load and changes in immune status during NA therapy are accompanied by decreased HBV-miR-3 levels, with HBV-miR-3 showing varying degrees of correlation with HBV DNA, HBV RNA, HBsAg, and HBeAg during early treatment.

We further plotted ROC curves for each indicator, calculated the Youden index for each value on the curves, and determined the optimal cutoff values. At baseline, when HBV-miR-3 ≤ 6.9572, the positive predictive value (PPV) reached 74.49%, which was comparable to that of HBV RNA and higher than that of HBsAg. At week 12 of treatment, the PPV was 73.33% when HBV-miR-3 ≤ 6.9048. At week 24, the PPV was 77.14% when HBV-miR-3 ≤ 4.6142. These results indicate that HBV-miR-3 at baseline, week 12, and week 24 has significance in predicting HBV DNA negativity at week 48 of NA treatment, with a predictive ability comparable to HBV RNA, slightly higher than HBsAg, but lower than HBeAg (Figure 4, Table 4). We further explored the potential of HBV-miR-3 in combined prediction. Multivariate regression and combined prediction analyses of the ability of HBV-miR-3, HBV DNA, HBV RNA, and HBsAg at weeks 0, 12, and 24 to predict HBV DNA negativity at week 48 revealed that the PPVs of HBV-miR-3 combined with HBV RNA for this prediction were 88.00%, 83.33%, and 88.89% at weeks 0, 12, and 24, respectively; those of HBV-miR-3 combined with HBsAg were 83.87%, 78.79%, and 86.96%. Notably, the PPVs of HBV-miR-3 combined with HBV DNA were the highest, reaching 85.19%, 100.00%, and 95.65% (Table 5). These findings demonstrate the ability of HBV-miR-3 as an independent and combined predictor of virological response.

DISCUSSION

Chronic HBV infection poses a major global health burden, with NAs being key therapeutic agents, though their efficacy evaluation remains critical. This randomized clinical trial investigated the predictive value of HBV-miR-3—an HBV-encoded microRNA with known roles in viral replication and pathogenesis—in NA therapy. Patients were randomized to TMF or TDF for 48 weeks, followed by TMF monotherapy up to 144 weeks. Analyses of virological indicators (HBV DNA, HBV RNA, HBsAg, HBeAg) and HBV-miR-3 revealed that HBV-miR-3 levels at baseline, week 12, and week 24 independently predicted HBV DNA

negativity at week 48, with PPVs comparable to HBV RNA and superior to HBsAg. Notably, combined prediction with HBV-miR-3 and HBV DNA yielded even higher PPVs (up to 100% at week 12). These findings highlight HBV-miR-3 as a promising independent and combinatorial marker for evaluating NA therapeutic efficacy in chronic hepatitis B.

HBV-miR-3, a key regulatory molecule encoded by the viral genome, exerts multiple roles in viral replication, immune evasion, and disease progression. As a functional microRNA encoded by the HBV genome, HBV-miR-3 has a highly conserved sequence and can be detected in both HBV-infected hepatocytes and peripheral blood exosomes. Notably, it is exclusively present in patients with chronic hepatitis B (CHB) and is not found in healthy individuals or non-HBV-infected populations[17,18]. Owing to the uniqueness of its structure and biological functions, HBV-miR-3 holds substantial clinical value as a potential indicator for predicting therapeutic efficacy in patients with chronic hepatitis B. Our previous study has demonstrated that HBV-miR-3 exhibits a strong predictive capacity for HBeAg seroconversion in HBV-infected patients receiving PegIFN-α therapy. This multi-center phase III study established HBV-encoded miR-3 as a novel replication biomarker positively correlating with HBV DNA, pgRNA, HBeAg, and HBsAg levels during PegIFN-α therapy in 650 $HBeAg^+$ patients. Patients with week-12 HBV-miR-3 <3 log10 copies/mL achieved 70.0% seroconversion versus 0% with levels >6 log10 copies/mL. Combining week-12 HBV-miR-3 and HBeAg enhanced predictive accuracy to 83.64%, supporting its utility for early treatment response monitoring[16]. In real-world clinical practice, NA therapy is far more widely used than PegIFN-α in treating patients with chronic hepatitis B. Thus, we aimed to further explore the predictive role and clinical translational potential of HBV-miR-3 in HBV-infected patients receiving NA therapy. Consistent with our hypothesis, the results of this study demonstrated that HBV-miR-3 levels at weeks 0, 12, and 24 all exhibited robust predictive value for virological response, with a predictive capacity comparable to that of HBV RNA. In combined prediction analyses, HBV-miR-3, when combined with HBV DNA, HBV RNA, or HBsAg, showed favorable combined predictive performance for virological response at week 48.

The strong predictive potential of HBV-miR-3 may be attributed to its direct regulation of viral replication. HBV-miR-3 binds to specific sequences in pgRNA, inhibiting the synthesis of core proteins and thereby reducing the release of viral particles. This process does not affect viral DNA polymerase activity, indicating a highly specific mode of action[10]. Furthermore, the robust predictive capacity of HBV-miR-3 may also relate to its targeted regulation of host genes. One study revealed that HBV-miR-3 promotes HCC cell proliferation by inhibiting the host protein phosphatase PPM1A, which relieves PPM1A-mediated suppression of the MAPK pathway[17]. Another study demonstrated that in HBV-related HCC, HBV-miR-3 targets the 3'-UTR of the hypoxia-inducible FIH-1, alleviating FIH-1-dependent inhibition of the HIF-1α/VEGFA pathway and promoting tumor angiogenesis[15]. By analogy, the targeted regulation of HBV by HBV-miR-3 might act through protein phosphorylation pathways and angiogenesis regulatory mechanisms. Additionally, remodeling of the immune microenvironment could represent another regulatory mechanism underlying HBV-miR-3's predictive role. Research has shown that HBV-miR-3 enhances the antiviral effect of IFN-α by activating the JAK/STAT pathway, promotes macrophage polarization toward the M1 phenotype, and strengthens antiviral immune responses[12]. In chronic HBV

infection, HBV-miR-3 may downregulate host immune-related genes (e.g., MHC-I molecules), reducing the sensitivity of hepatocytes to cytotoxic T cells and thereby facilitating immune evasion[19].

NAs cannot eliminate cccDNA within hepatocytes. HBV cccDNA is characterized by high stability, with its levels remaining largely constant in hepatocytes. Even when HBV DNA and HBsAg become undetectable during treatment, cccDNA can still be detected in patients' hepatocytes, indicating a risk of viral reactivation[20,21]. Targeted clearance of cccDNA during therapy is critical for curing chronic HBV infection. Liver biopsy is currently the only reliable method for detecting cccDNA; however, as an invasive procedure, it carries risks of bleeding and infection in patients, involves a relatively long turnaround time for results, and is thus unsuitable as a routine indicator for monitoring viral activity in chronic HBV-infected individuals[22]. HBV-miR-3 can serve as a surrogate marker for cccDNA, as its serum levels are significantly correlated with the activity of cccDNA in liver tissues. Notably, the detection of exosomal HBV-miR-3 is non-invasive, making it suitable for long-term monitoring and therapeutic efficacy evaluation. It is noteworthy that in our study, patients in the DNA-negative group were significantly older at baseline than those in the DNA-positive group, with baseline levels of HBV RNA, HBV DNA, HBV-miR-3, and HBeAg all significantly lower than those in the DNA-positive group at week 48. This indicates distinct differences in the viral activity and the patients' own immune status at the initiation of treatment. Furthermore, an interesting observation was that after 144 weeks of treatment, the HBV DNA negativity rate reached 90.6%, whereas only 52.8% of patients achieved HBV-miR-3 negativity. Previous studies have noted that the high rate of virological relapse after discontinuation is attributable to the need for long-term NA therapy in most patients[23]. These findings suggest that due to the difficulty in completely eliminating cccDNA, persistent expression of HBV-miR-3 may occur even when HBV DNA is undetectable in a single test. This implies that HBV-miR-3 could serve as an effective indicator for continued monitoring of viral activity and long-term antiviral efficacy after HBV DNA negativity during prolonged NA therapy.

Our study has several limitations that warrant improvement. First, after screening patients using strict inclusion and exclusion criteria, the final number of enrolled patients was relatively small. Nevertheless, our findings do demonstrate clinically meaningful implications. In the future, we will enroll more patients to validate these results, aiming to enhance the generalizability of our conclusions. Second, although our study extended the follow-up period to 144 weeks—longer than most existing clinical studies (which typically last 96 weeks)—long-term cohort studies are still needed to clarify the association between HBV-miR-3 levels and end-stage liver diseases such as cirrhosis and hepatocellular carcinoma. Third, current research has primarily focused on HBV genotypes B and C. Future studies should explore and validate the expression patterns of HBV-miR-3 and its impact on treatment response in other genotypes (e.g., genotype D). Fourth, our previous work and the present study respectively identified HBV-miR-3 as a reliable predictor of virological response (HBV DNA) during PegIFN-α and NA therapy. Going forward, we will focus on the potential application of HBV-miR-3 in combination therapy strategies. We hypothesize that combining NA therapy with PegIFN-α may more effectively reduce HBV-miR-3 levels and promote HBsAg clearance through dual mechanisms: direct inhibition of viral replication coupled with immune activation.

CONCLUSION

HBV-miR-3 is closely associated with hepatitis B virus replication and serves as a robust predictor of HBV-DNA virological response during NA therapy. It also exhibits potential as a marker for viral activity levels and a promising therapeutic target in the context of antiviral treatment.

Declarations

Institutional review board statement: The research was approved by the Ethics Committee of the Second Xiangya Hospital.

Clinical trial registration statement: This trial was registered at http: //clinicaltrials.gov as (NCT03903796).

Informed consent statement: All study participants or their legal guardian provided informed written consent regarding personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: The authors disclose no conflicts.

Data sharing statement: The data that support the findings of this study are available in https://clinicaltrials.gov/study/NCT03903796?term=NCT03903796&rank = 1 .

CONSORT 2010 statement: The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.

Author contributions: Z. D. and Z. Z. wrote the main manuscript text and prepared figures and tabs. Both of them are first authors. M. Z. and H. Z. designed the study. Z. D. is responsible for data collecting. Y. Y.and Z.Y analyzed all data. All authors reviewed the manuscript.

FundingThis study was supported by Key Program of the National Natural Science Foundation of China (82430071 to H.Z.).

ACKNOWLEDGEMENTS

We would like to thank all participants for collecting the data of this study.

We declare that the authors have no conflict of interest.

The data that support the findings of this study are available in https://clinicaltrials.gov/study/NCT03903796?term=NCT03903796&rank = 1.

References

1. GUIDOTTI L G, CHISARI F V. Immunobiology and pathogenesis of viral hepatitis[J]. Annu Rev Pathol, 2006,1: 23-61.
2. HSU Y C, HUANG D Q, NGUYEN M H. Global burden of hepatitis B virus: current status, missed opportunities and a call for action[J]. Nat Rev Gastroenterol Hepatol, 2023,20(8): 524-537.
3. MENENDEZ-ARIAS L, ALVAREZ M, PACHECO B. Nucleoside/nucleotide analog inhibitors of hepatitis B virus polymerase: mechanism of action and resistance[J]. Curr Opin Virol, 2014,8: 1-9.
4. YOU H, WANG F, LI T, et al. Guidelines for the Prevention and Treatment of Chronic Hepatitis B (version 2022)[J]. J Clin Transl Hepatol, 2023,11(6): 1425-1442.
5. ZHANG Q, LIANG J, YIN J, et al. Real-life impact of tenofovir disoproxil fumarate and entecavir therapy on lipid profile, glucose, and uric acid in chronic hepatitis B patients[J]. J Med Virol, 2022,94(11): 5465-5474.
6. LIU Z, JIN Q, ZHANG Y, et al. Randomised clinical trial: 48 weeks of treatment with tenofovir amibufenamide versus tenofovir disoproxil fumarate for patients with chronic hepatitis B[J]. Aliment Pharmacol Ther, 2021,54(9): 1134-1149.
7. BYUN K S, CHOI J, KIM J H, et al. Tenofovir Alafenamide for Drug-Resistant Hepatitis B: A Randomized Trial for Switching FromTenofovir Disoproxil Fumarate[J]. Clin Gastroenterol Hepatol, 2022,20(2): 427-437.
8. NASSAL M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B[J]. Gut, 2015,64(12): 1972-1984.
9. VACHON A, SEO G E, PATEL N H, et al. Hepatitis B virus serum RNA transcript isoform composition and proportion in chronic hepatitis B patients by nanopore long-read sequencing[J]. Front Microbiol, 2023,14: 1233178.
10. YANG X, LI H, SUN H, et al. Hepatitis B Virus-Encoded MicroRNA Controls Viral Replication[J]. J Virol, 2017,91(10).
11. YAN Y, CHEN S, LIAO L, et al. ALV-miRNA-p19-01 Promotes Viral Replication via Targeting Dual Specificity Phosphatase 6[J]. Viruses, 2022,14(4).
12. ZHAO X, SUN L, MU T, et al. An HBV-encoded miRNA activates innate immunity to restrict HBV replication[J]. J Mol Cell Biol, 2020,12(4): 263-276.
13. XU Z Y, GAO J S, HE Y, et al. Hepatitis B virus confers innate immunity evasion through hepatitis B virus-miR-3 down-regulation of cGAS-Sting-IFN signaling[J]. World J Hepatol, 2025,17(2): 99292.
14. TANG J, XIAO X, JIANG Y, et al. miR-3 Encoded by Hepatitis B Virus Downregulates PTEN Protein Expression and Promotes Cell Proliferation[J]. J Hepatocell Carcinoma, 2020,7: 257-269.
15. CHEN H, CAO D, HAN N, et al. Hepatitis B Virus-Encoded MicroRNA (HBV-miR-3) Inhibits FIH-1 Expression to Promote Tumor Angiogenesis in HBV-Related Hepatocellular Carcinoma[J]. J Hepatocell Carcinoma, 2023,10: 2337-2353.
16. XU Z, XU Y, WU Z, et al. HBV-miR-3 is closely related to HBV replication and strongly predictive of HBeAg seroconversion in PegIFN-alpha treated patients[J]. Sci Rep, 2024,14(1): 1502.

17. Chavalit T, Nimsamer P, Sirivassanametha K, et al. Hepatitis B Virus-Encoded MicroRNA (HBV-miR-3) Regulates Host Gene PPM1A Related to Hepatocellular Carcinoma. Microrna. 2020;9(3):232-239. doi:10.2174/2211536608666191104105334
18. Gan W, Chen X, Wu Z, et al. The relationship between serum exosome HBV-miR-3 and current virological markers and its dynamics in chronic hepatitis B patients on antiviral treatment. Ann Transl Med. 2022;10(10):536. doi:10.21037/atm-22-2119
19. Taylor BC, Balko JM. Mechanisms of MHC-I Downregulation and Role in Immunotherapy Response. Front Immunol. 2022;13:844866. Published 2022 Feb 28. doi:10.3389/fimmu.2022.844866
20. Lin YH, Lai MW, Yeh CT, Lin WR. Perspectives on NcRNAs in HBV/cccDNA-driven HCC progression. Cancer Cell Int. 2025;25(1):224. Published 2025 Jun 21. doi:10.1186/s12935-025-03849-0
21. Diogo Dias J, Sarica N, Neuveut C. Early Steps of Hepatitis B Life Cycle: From Capsid Nuclear Import to cccDNA Formation. Viruses. 2021;13(5):757. Published 2021 Apr 26. doi:10.3390/v13050757
22. Barnabas A, Miquel R. A different picture on liver biopsy. J Hepatol. 2024;80(5):e189-e191. doi:10.1016/j.jhep.2024.01.038
23. HALL S, VOGRIN S, WAWRYK O, et al. Discontinuation of nucleot(s)ide analogue therapy in HBeAg-negative chronic hepatitis B: a meta-analysis[J]. Gut, 2022,71(8): 1629-1641.

Tables

Table1 Baseline demographic and virological profiles and characteristics of enrolled patients

	All Patients(n=53)	TDF Treatment(n=14)	TDF Treatment(n=39)	*p*-value
Sex (Male/Female)	36/17	8/6	28/11	0.314
Age (years)	36.89±1.44	34.50±1.95	37.74±1.83	0.327
BMI(kg/m^2)	22.83±0.67	22.01±1.02	23.16±0.85	0.448
WBC(10^9)	6.13±0.22	6.27±0.45	6.08±0.26	0.715
Hgb(g/L)	149.02±2.06	147.79±3.88	149.46±2.45	0.723
Plt(10^9)	214.62±10.01	234.00±15.58	207.67±12.31	0.250
ALT(IU/mL)	129.10±17.09	176.42±53.02	112.12±13.01	0.097
AST(IU/mL)	79.79±9.70	99.85±28.31	73.10±8.91	0.236
HBV-RNA	4.14±0.39	3.99±0.86	4.18±0.44	0.845
HBV-DNA	6.81±0.17	7.20±0.28	6.67±0.21	0.181
HBV-miR-3	5.38±0.29	5.33±0.60	5.40±0.34	0.919
HBsAg	2.94±0.12	3.12±0.25	2.88±0.14	0.381
HBeAg	0.98±0.27	1.33±0.54	0.85±0.31	0.441

BMI:Body Mass Index; WBC:White Blood Cel; Hgb:Hemoglobin; Plt:Platelet; ALT:Alanine Aminotransferase; AST:Aspartate Aminotransferase; HBV-RNA:Hepatitis B Virus Ribonucleic Acid; HBV-DNA:Hepatitis B Virus Deoxyribonucleic Acid; HBV-miR-3: HBV-encoded microRNA-3; HBsAg:Hepatitis B surface Antigen; HBeAg:Hepatitis B e Antigen. The log10 transformation was performed prior to analysis. Biomarker units are measured by log10 copies/mL for HBV-miR-3 and HBV RNA, log10 IU/mL for HBV DNA, log10 IU/mL for HBsAg, and log10 COI for HBeAg.

Table2 Baseline demographic and virological characteristics related to DNA- and DNA+ groups at week 48

	All patients(n=53)	DNA+ (n=19)	DNA- (n=34)	*p*-value
Sex (Male/Female)	36/17	13/6	23/11	0.954
Age (years)	36.89±1.44	32.16±2.28	39.53±1.72	0.013
BMI(kg/m^2)	22.83±0.67	21.12±0.79	23.52±0.83	0.106
WBC(10^9)	6.13±0.22	6.03±0.27	6.19±0.32	0.736
Hgb(g/L)	149.02±2.06	148.58±3.98	149.26±2.36	0.875
Plt(10^9)	214.62±10.01	232.79±19.14	204.47±11.21	0.177
ALT(IU/mL)	129.10±17.09	111.42±14.75	138.99±25.35	0.445
AST(IU/mL)	79.79±9.70	76.59±15.09	81.63±12.74	0.805
HBV-RNA	4.14±0.39	5.93±0.36	3.21±0.47	0.000
HBV-DNA	6.81±0.17	7.52±0.23	6.41±0.21	0.002
HBV-miR-3	5.38±0.29	6.54±0.29	4.73±0.39	0.002
HBsAg	2.94±0.12	2.84±0.24	3.00±0.13	0.513
HBeAg	0.98±0.27	2.14±0.36	0.33±0.32	0.001

BMI:Body Mass Index; WBC:White Blood Cel; Hgb:Hemoglobin; Plt:Platelet; ALT:Alanine Aminotransferase; AST:Aspartate Aminotransferase; HBV-RNA:Hepatitis B Virus Ribonucleic Acid; HBV-DNA:Hepatitis B Virus Deoxyribonucleic Acid; HBV-miR-3: HBV-encoded microRNA-3; HBsAg:Hepatitis B surface Antigen; HBeAg:Hepatitis B e Antigen. The log10 transformation was performed prior to analysis. Biomarker units are measured by log10 copies/mL for HBV-miR-3 and HBV RNA, log10 IU/mL for HBV DNA, log10 IU/mL for HBsAg, and log10 COI for HBeAg.

Table 3□Chi-square test for HBV DNA and HBV-miR-3 clearance at week 144

Indicator at week 144	Total	Cleared	Not cleared	P□0□1□
HBV-DNA	53	5	48	
HBV-miR-3	47(6 LTFU)	25	22	
HBV-miR-3 in DNA+ subjects	4(1 LTFU)	4	0	0.0498
HBV-miR-3 in DNA- subjects	43(5 LTFU)	21	22	

HBV-DNA:Hepatitis B Virus Deoxyribonucleic Acid; HBV-miR-3: HBV-encoded microRNA-3.

Table 4 Cutoff values, positive predictive value (PPV), and negative predictive value (NPV) for predicting HBV DNA clearance at week 48

	Cutoff(log10)	PPV	NPV
0w			
HBV-miR-3	≤6.9572	79.49%	78.57%
HBV RNA	≤5.4106	84.62%	66.67%
HBsAg	≤2.5105	73.68%	60.00%
HBeAg	≤-0.2757	90.91%	54.84%
12w			
HBV-miR-3	≤6.9048	73.33%	100%
HBV RNA	≤3.3469	84.00%	62.50
HBsAg	≤3.3562	72.97%	56.25%
HBeAg	≤-0.3279	88.00%	57.14%
24w			
HBV-miR-3	≤4.6142	77.14%	61.11%
HBV RNA	≤2.8009	87.50%	64.71%
HBsAg	≤3.1826	74.19%	50.00%
HBeAg	≤0.0755	85.71%	60.00%

PPV: positive predictive value; NPV: negative predictive value; HBV-DNA:Hepatitis B Virus Deoxyribonucleic Acid; HBV-miR-3: HBV-encoded microRNA-3; HBV-RNA:Hepatitis B Virus Ribonucleic Acid; HBsAg:Hepatitis B surface Antigen; HBeAg:Hepatitis B e Antigen.

Table 5 Joint predictors for HBV DNA clearance at week 48 for HBV DNA clearance at week 48

	PPV	NPV
0w		
HBV-miR-3+HBV RNA	88.00%	87.50%
HBV-miR-3+HBsAg	83.87%	85.71%
HBV-miR-3+HBV DNA	85.19%	90.91%
12w		
HBV-miR-3+HBV RNA	83.33%	100.00%
HBV-miR-3+HBsAg	78.79%	100.00%
HBV-miR-3+HBV DNA	100.00%	100.00%
24w		
HBV-miR-3+HBV RNA	88.89%	77.78%
HBV-miR-3+HBsAg	86.96%	60.00%
HBV-miR-3+HBV DNA	95.65%	78.57%

PPV: positive predictive value; NPV: negative predictive value; HBV-DNA:Hepatitis B Virus Deoxyribonucleic Acid; HBV-miR-3: HBV-encoded microRNA-3; HBV-RNA:Hepatitis B Virus Ribonucleic Acid; HBsAg:Hepatitis B surface Antigen; HBeAg:Hepatitis B e Antigen.

Figures

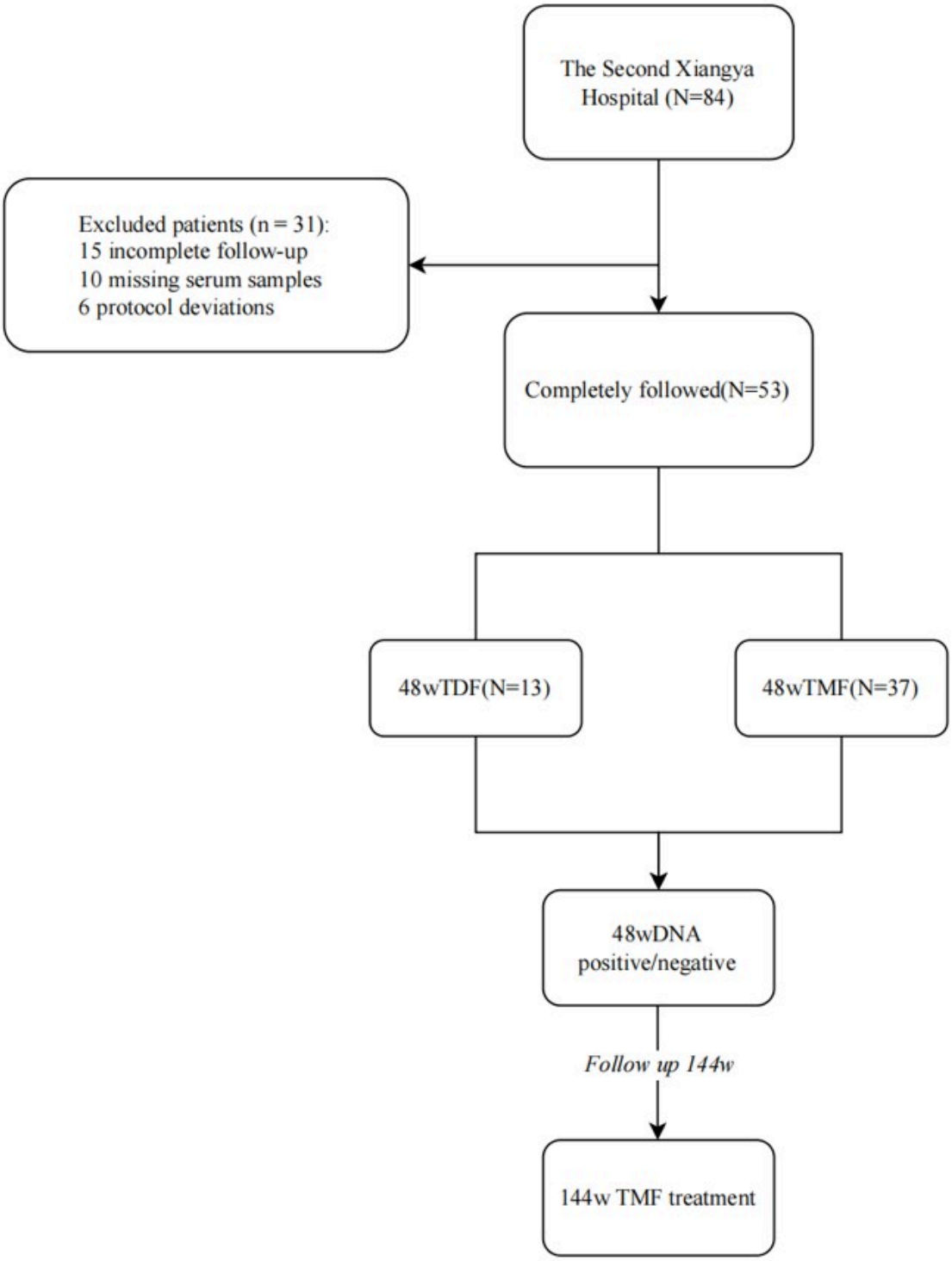


Figure 1

Flow diagram.

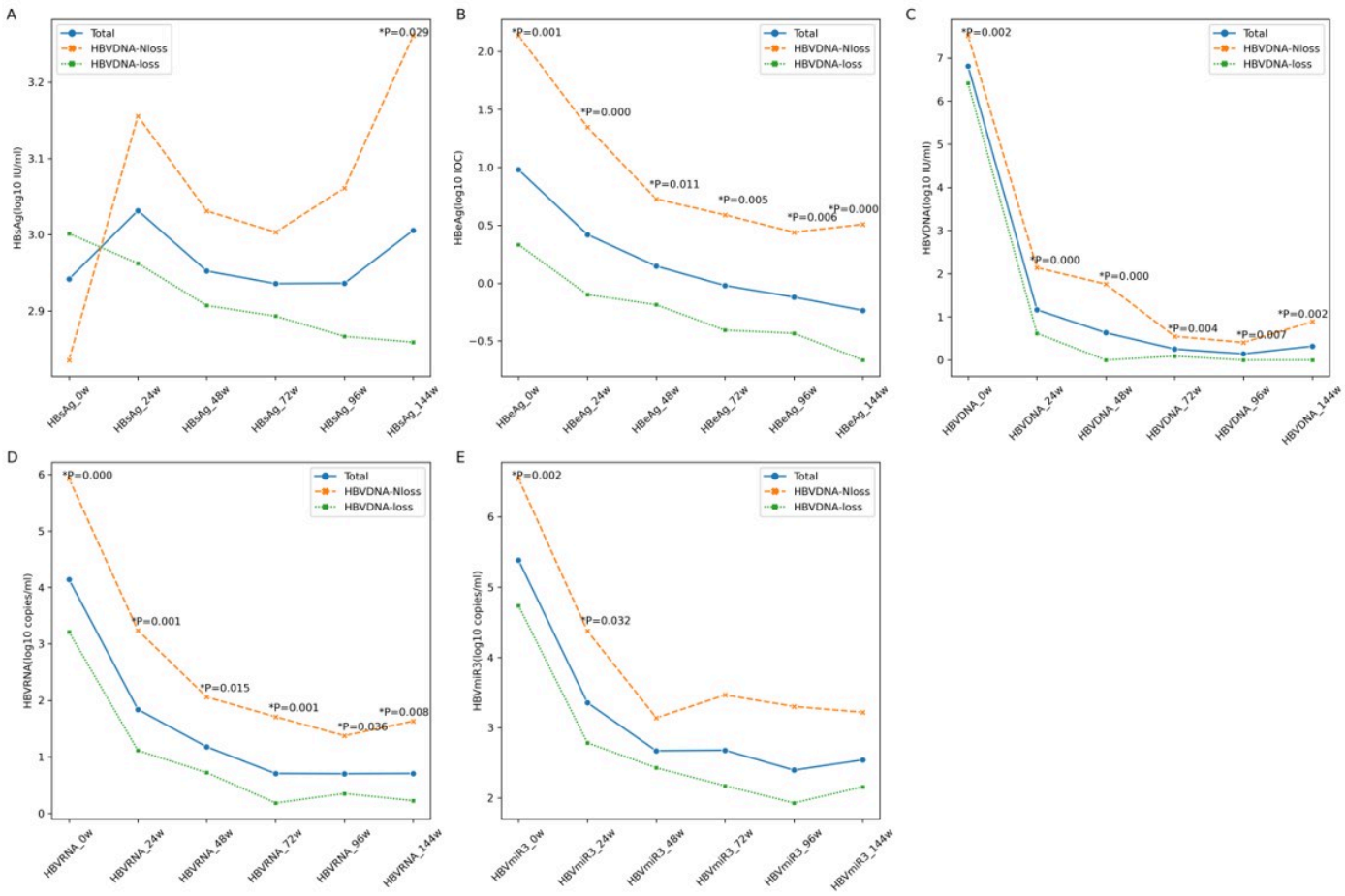


Figure 2

Dynamic changes of HBV biomarkers in DNA-positive group, DNA-negative group, and all patients during 48w treatment. A: HBsAg; B: HBeAg; C: HBV DNA; D: HBV RNA; E: HBV-miR-3

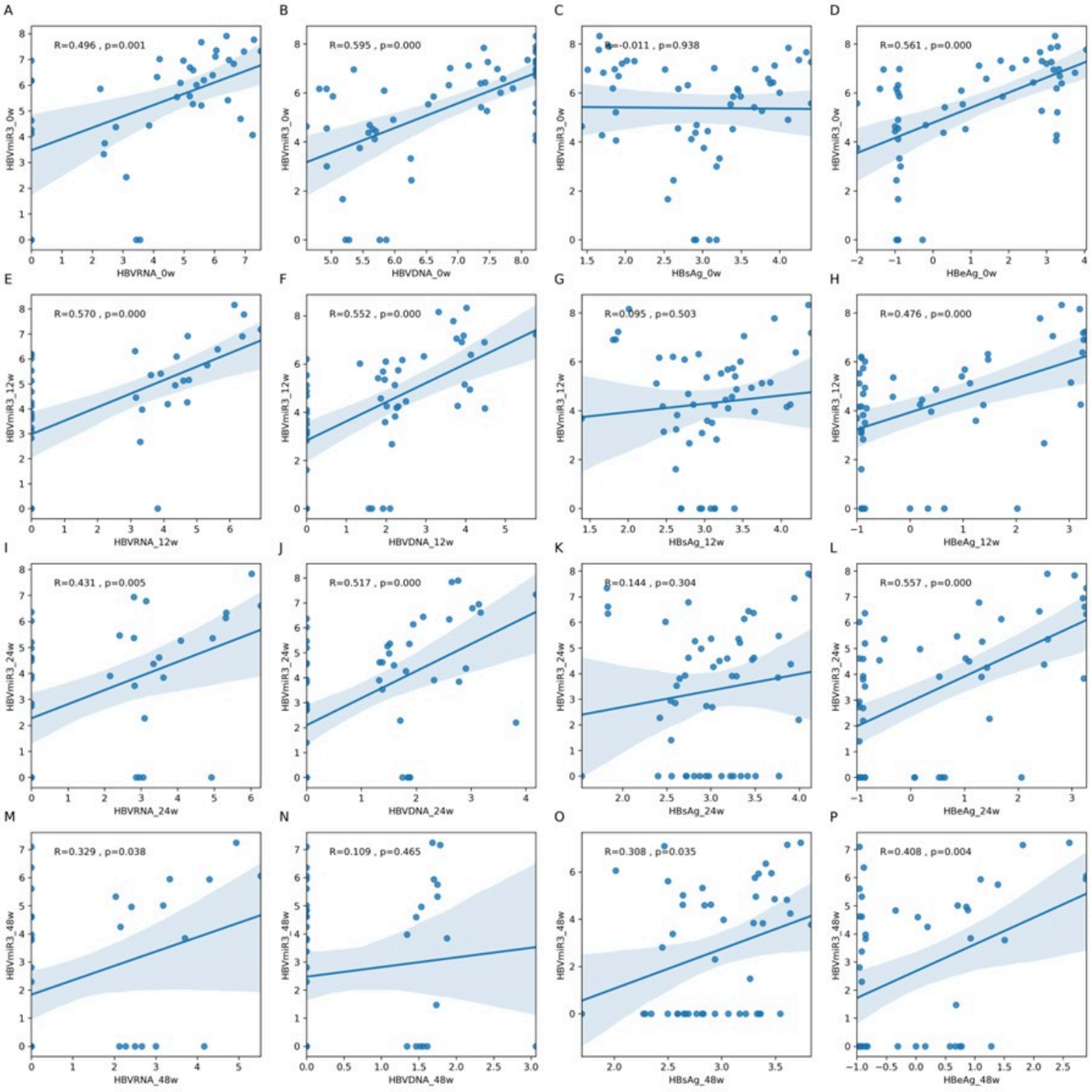


Figure 3

Correlations between HBV-miR-3 and other viral biomarkers at weeks 0, 12, 24, and 48

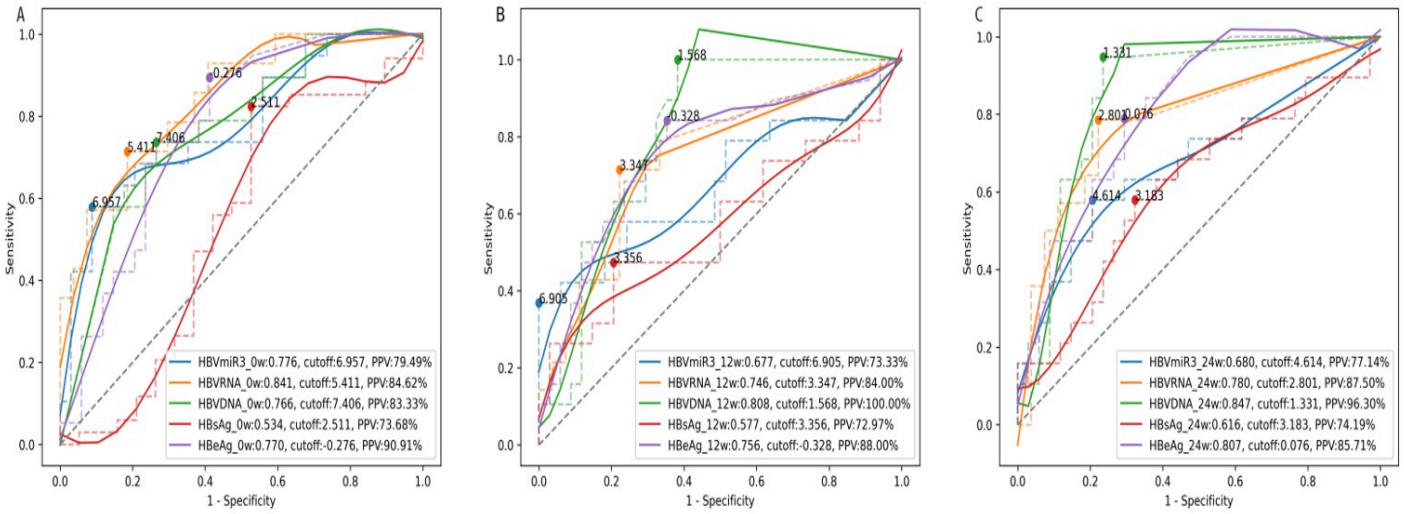


Figure 4

ROC curves of viral biomarkers at weeks 0 (A), 12 (B), and 24 (C) for predicting HBV DNA clearance

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- summary.pdf