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ORIGINAL ARTICLE

Serum miR-181b Is Correlated with Hepatitis B Virus Replication and Disease Progression in Chronic Hepatitis B Patients

Fujun Yu[1] · Guangyao Zhou[2] · Guojun Li[3] · Bicheng Chen[4] · Peihong Dong[1] ·
Jianjian Zheng[4] (iD)

Abstract

Background Previously, we reported that microRNA-181b (miR-181b) activates hepatic stellate cells partly through the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/Akt pathway.

Aims The main objective of this study was to ascertain whether serum miR-181b expression is correlated with that of liver hepatitis B virus (HBV) DNA and disease progression in chronic hepatitis B (CHB) patients.

Methods Serum miR-181b and liver HBV DNA levels were quantified in 64 CHB patients with real-time PCR. Liver fibrosis and necroinflammation were graded according to the Ishak scoring system.

Results Serum miR-181b levels were evaluated in the CHB group, compared with healthy controls. Expression in patients with HBsAg (+) was higher than that in patients with HBsAg (−). Notably, serum miR-181b and liver HBV DNA levels were significantly correlated ($P < 0.05$). Serum miR-181 levels were higher in patients with serum HBV DNA > 10^3 IU/ml ($P = 0.017$), histologic activity index (HAI) >8 ($P = 0.001$) and fibrosis score >4 ($P < 0.0001$). Liver HBV DNA levels were higher in patients with abnormal alanine aminotransferase (ALT) values ($P = 0.004$), serum HBV DNA levels > 10^3 IU/ml ($P = 0.034$) and fibrosis score >4 ($P = 0.006$). Using multivariate logistic regression analysis, serum miR-181b was identified as an independent predictor of disease progression (OR 4.172, 95 % CI 1.838–9.473, $P = 0.009$ for HAI >8; OR 5.387, 95 % CI 2.067–14.036, $P = 0.001$ for fibrosis score >4).

Conclusions Serum miR-181b is correlated with liver and serum HBV DNA levels as well as disease progression in CHB.

Keywords Serum microRNA-181b · Liver fibrosis · Chronic hepatitis B · Biomarker

✉ Peihong Dong
dongpeihong111@163.com

✉ Jianjian Zheng
120378196@qq.com

1   Department of Infectious Diseases, The First Affiliated Hospital of Wenzhou Medical University, No. 2 FuXue Lane, Wenzhou 325000, Zhejiang, People's Republic of China

2   Department of Infectious Diseases, The Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University, Wenzhou 325000, People's Republic of China

3   Department of Hepatology, The Second Yinzhou Hospital, Ningbo 315000, People's Republic of China

4   Wenzhou Key Laboratory of Surgery, The First Affiliated Hospital of Wenzhou Medical University, No. 2 FuXue Lane, Wenzhou 325000, Zhejiang, People's Republic of China

Introduction

Chronic hepatitis B virus (HBV) affects 350–400 million patients worldwide and is one of the major etiological factors for the development of cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. The HBV DNA level has been identified as one of the risk factors of cirrhosis and HCC for chronic hepatitis B (CHB) patients [3]. In China, HBV infection is a predominant etiological factor of liver disease, whereby ∼7.8 % of the population are HBV carriers [4]. China is additionally one of the areas with the highest prevalence of HCC due to the significant number of chronic HBV carriers [5]. CHB poses a serious health burden, leading to increased healthcare costs and other socioeconomic problems. Liver biopsy has been reported to be valuable in the assessment of HBV-induced liver injury

severity and monitoring of CHB progression [6]. However, due to the invasive procedure and possible complications, such as bleeding and sampling error, this technique is not commonly used in CHB patients. Therefore, the identification of potential effective non-invasive markers of CHB progression is critical.

MicroRNAs (miRNAs) are small non-coding RNAs (18–24 nucleotides long) that modulate gene expression by binding to the 3′-untranslated region of target mRNAs [7]. MiRNAs are involved in several physiological processes, including cellular differentiation, apoptosis and proliferation [8], and play vital roles in pathogenesis, diagnosis and therapeutic aspects of viral infection [9, 10]. Increasing evidence has shown that miRNAs act as HSC regulators and play diverse roles in hepatic fibrosis [11]. Moreover, cell-free miRNAs circulate in the blood and are detectable in serum and plasma in a sufficiently stable form to serve as biomarkers [12–15]. The miRNA levels in serum and plasma are strongly correlated, indicating that both serum and plasma may be employed for the analysis of these blood-based biomarkers [14]. Altered levels of serum/plasma miRNAs are associated with numerous diseases, including cancer and liver disease [16–18]. Thus, circulating miRNAs have significant potential as non-invasive markers for human liver fibrosis.

Previously, Wang et al. [11] reported that miR-181b, but not miR-181a, promotes hepatic stellate cell proliferation by targeting p27, and is elevated in serum of cirrhosis patients. Our group further demonstrated that miR-181b activates hepatic stellate cells (HSCs), at least in part, via the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/Akt pathway [19]. Overexpression of miR-181b resulted in enhanced cell proliferation, and expression of ECM proteins and α-SMA in HSCs. However, the issue of whether serum miR-181b expression is associated with HBV replication and CHB disease progression remains to be established. In the present study, we investigated the serum levels of miR-181b and its possible correlation with serum HBV DNA and progression of liver fibrosis in CHB patients.

Materials and Methods

Study Subjects

Serum samples were obtained from patients attending the First Affiliated Hospital of Wenzhou Medical University in Wenzhou and the Second Yinzhou Hospital in Ningbo from 2007 to 2012 (Table 1). The study included 72 healthy controls (with normal liver biochemistry, no history of liver disease or alcohol abuse, and no viral hepatitis) and 72 patients with CHB. Patients with CHB who were naïve to nucleos(t)ide analogues and interferon

therapy were included. All patients were hepatitis B surface antigen (HBsAg)-positive for at least 6 months. Exclusion criteria were: (1) patients aged less than 16 years, (2) co-infection with human immunodeficiency virus (HIV), (3) coexistence of liver injury caused by other etiologies, including hepatitis C virus (HCV) infection, drug intake, alcohol consumption and auto-immune hepatitis, (4) severe systematic diseases, and (5) pregnancy and lactation [20]. Eight patients were excluded owing to coagulation disorders. Overall, 64 patients underwent liver biopsy. Demographic and clinical information were obtained, and blood samples collected from all patients on the day of diagnostic liver biopsy. Diagnosis of HCC was based on histopathological assessment [21].

Ethics Statement

Informed consent for the use of blood samples was obtained from all participants. The project was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All procedures were performed in accordance with the current international guidelines, standards on human experimentation of the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University, and the Helsinki Declaration of 1975, revised in 1983.

Liver Histology

Liver biopsy was performed using a 16-gauge Menghini needle. Each liver biopsy case was advised by physicians in care, and liver specimens at least in 2.0 cm in length obtained. Samples were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin. Results were reviewed by experienced hepatopathologists. In addition, at least 8–10 portal tracts in samples are required to admit patients. Histological activity index (HAI) and fibrosis stages (F0 = no fibrosis to F6 = cirrhosis) were assessed according to the Ishak scoring system [22].

Blood Samples Processing

All blood samples were centrifuged at $3400g$ for 7 min at room temperature within 4 h of acquisition. Sera were transferred into Eppendorf tubes and additionally centrifuged at $12,000g$ for 10 min at 4 °C to remove the remaining cells. Serum samples were stored at −80 °C pending RNA extraction.

Detection of miR-181b with Real-Time Quantitative RT-PCR

Total RNA was extracted with the miRNeasy Mini Kit (Qiagen, Carlsbad, CA, USA), according to the

Table 1 Characteristics of CHB patients and healthy subjects

	CHB patients ($n = 64$)	Healthy subjects ($n = 72$)
Age (years), mean ± SD	41.3 ± 9.2	42.8 ± 12.9
Sex (n)		
Male	37 (57.8 %)	43 (59.7 %)
Female	27 (42.2 %)	29 (40.3 %)
ALT (U/L), mean ± SD	103.2 ± 115.5	
Serum miR-181b, mean ± SD	2.688 ± 1.097	
Liver HBV DNA (IU/cells), mean ± SD	2.282 ± 4.499	
Serum HBV DNA (IU/mL), mean ± SD	$1.6 \times 10^6 \pm 9.9 \times 10^6$	
HBeAg		
+	28 (43.8 %)	
−	36 (56.2 %)	
HAI (n)		
2	5 (7.8 %)	
3	17 (26.6 %)	
4	3 (4.7 %)	
5	6 (9.4 %)	
6	5 (7.8 %)	
7	8 (12.5 %)	
8	5 (7.8 %)	
9	6 (9.4 %)	
≥11	9 (14.0 %)	
Fibrosis (n)		
F0	4 (6.3 %)	
F1	15 (23.4 %)	
F2	8 (12.5 %)	
F3	5 (7.8 %)	
F4	7 (10.9 %)	
F5	20 (31.3 %)	
F6	5 (7.8 %)	

manufacturer's instructions for liquid samples. For removing DNA contaminants in total RNA, DNase treatment (Qiagen) was performed. The final elution volume was 20 μl. Prior to the reverse transcription reaction, serum RNA preparations were quantified using NanoDrop 1000 (Nanodrop, Wilmingtion, DE, USA). Reverse transcription was performed in a 20 μl reaction volume using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). For cDNA synthesis, reaction mixtures were sequentially incubated at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. miR-181b was quantified in triplicate via qRT-PCR using the TaqMan MicroRNA Assay Kit (Applied Biosystems). According to the standard TaqMan MicroRNA assay protocol, real-time PCR was performed on an ABI 7500 Real-Time PCR system (Applied

Biosystems) under the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Each PCR mixture (20 μl) included the reverse transcription products, TaqMan 2X Universal PCR Master Mix without UNG Amperase, miRNA-specific TaqMan probes, and primers supplied by Applied Biosystems. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. Ct values were calculated with SDS 2.0.1 software (Applied Biosystems). The formula $2^{-\Delta Ct}$ was used to calculate the miRNA levels in serum, where $\Delta Ct = $ mean (Ct of internal references) − Ct of target miRNA. The relative expression levels of miR-181b were calculated and normalized to miR-16 (Applied Biosystems) using the comparative ΔCt method and the Eq. $2^{-\Delta Ct}$, as described previously [23].

Liver HBV DNA and Serum Hepatitis Markers Analysis

Liver tissues from biopsies were homogenized with TissueLyser (Qiagen, Hilden, Germany) for 30 s at 30 Hz, and DNA extracted using microspin columns (QIAamp DNA kit; Qiagen). DNA was analyzed for HBV genome levels with real-time PCR in a Light Cycler 1.5 (Roche Diagnostics, Branchburg, NJ, USA). An external standard curve was generated to quantify HBV genome levels using a PCR product cloned with the TA cloning system as standard (Invitrogen, Carlsbad, CA, USA), with 8 μl appropriately diluted plasmid used to generate the standard curve. HBV DNA was normalized to β-globin as a positive control, which is ubiquitously detected in all human cells [24]. Data are indicated as the number of IU/cells. Each value represents mean ± SD of three experiments.

For all CHB patients, HBsAg, HBeAg, and antibodies against HBsAg (anti-HBs), HBeAg (anti-HBe) and hepatitis B core antigen (anti-HBc) were determined using the Roche Modular E170 Immunoassay Analyzer (Roche, Basel, Switzerland). Serum HBV DNA was quantified with the artus HBV QS-RGQ Kit (Qiagen) using a lower detection limit of 10.2 IU/ml.

Statistical Analysis

Statistical analyses were performed with SPSS 13.0 (IBM, Armonk, NY, USA). Continuous variables were summarized as mean and standard deviation, and categorical variables as absolute and relative frequencies. Differences were evaluated using the Wilcoxon signed-rank test, and categorical variables compared with the Chi square test using the exact procedures, where required. Correlation coefficients (r) were calculated using Spearman's rank correlation. Odds ratios with 95 % confidence intervals

(CI) were estimated with a logistic regression model for evaluating the relationships among age, serum miR-181b, serum HBV DNA, HAI and fibrosis. Data were considered significant at $P < 0.05$.

Results

A total of 136 subjects were recruited, including 72 healthy controls and 64 CHB patients. Patient characteristics are shown in Table 1. We observed no significant differences in age between healthy controls and patients with CHB ($P = 0.746$). Sex distribution (male:female) was 43:29 in healthy controls and 37:27 in CHB patients ($P = 0.821$, χ^2 test).

Serum miR-181b in CHB patients was detected at a mean concentration of 2.688 ± 1.097 (Table 1). To ascertain its potential as a novel biomarker of CHB, serum miR-181b levels were detected in both CHB patients ($n = 64$) and healthy controls ($n = 72$). qRT-PCR analysis revealed higher serum miR-181b levels in CHB patients (median and range 2.550, 0.820–5.810), compared to healthy controls (0.920, 0.450–1.720; $P < 0.0001$, Fig. 1a). Liver HBV DNA was detected in CHB patients at a mean concentration of 2.282 ± 4.499 (Table 1). All CHB patients were divided into two subgroups according to HBeAg status. To determine the association between serum miR-181b and HBV replication, serum miR-181b levels were analyzed in HBeAg+ and HBeAg− groups. The serum miR-181b level in the HBeAg + group (median and range 2.905, 0.820–5.810) was significantly higher than that in the HBeAg- group (2.315, 0.870–4.700; $P < 0.0001$; Fig. 1b). CHB patients were additionally divided into two subgroups according to mean liver HBV DNA concentration. Our results showed that serum miR-181b levels in patients with liver HBV DNA ≤2.282 IU/cell (median and range 2.295, 0.820–5.300) are lower than those in patients with liver HBV DNA ≥2.282 IU/cell (3.570, 2.600–5.810; $P < 0.0001$; Fig. 1c). Furthermore, serum miR-181b and liver HBV DNA levels were correlated ($r = 0.595$, $P < 0.05$; Fig. 1d).

Serum miR-181b and liver HBV DNA levels were analyzed according to patient age, sex, alanine aminotransferase (ALT) values, HBV DNA serum levels, HAI and fibrosis scores. In univariate analysis, no association was found between serum miR-181b level and sex, age or ALT values. Notably, serum miR-181b levels were significantly increased in patients with HBV DNA serum levels $>10^3$ IU/ml [median 2.770 (1.890–4.700) vs. 2.210 (0.820–5.810), $P = 0.017$], HAI >8 [median 3.450 (1.890–5.810) vs. 2.420 (0.820–4.700), $P = 0.001$] and fibrosis scores >4 [median 3.320 (1.890–5.810) vs 2.220 (0.820–4.700), $P < 0.0001$] (Table 2). In terms of liver

HBV DNA, no association was evident with age, sex or HAI (Table 2). However, liver HBV DNA levels were significantly increased in patients with abnormal ALT values [median 2.485 (0.002–20.540) vs. 0.113 (0.006–2.140) (0.820–5.810), $P = 0.004$], HBV DNA serum level $>10^3$ IU/ml [median 0.716 (0.014–20.540) vs. 0.135 (0.002–2.310), $P = 0.034$] and fibrosis score >4 [median 1.100 (0.006–14.870) vs. 0.098 (0.002–20.540), $P = 0.006$] (Table 2).

To further confirm the association between serum miR-181b and histological lesions (HAI and fibrosis scores) and avoid possible confounding effects of other factors, such as age and liver HBV DNA levels, multivariate logistic regression analysis was performed. Serum miR-181b was identified as an independent predictor of HAI >8 (OR 4.172, 95 % CI 1.838–9.473, $P = 0.009$) and of a fibrosis score >4 (OR 5.387, 95 % CI 2.067–14.036, $P = 0.001$) (Table 3). Increasing HAI or fibrosis scores were accompanied by a progressive increase in the mean serum miR-181b level (Fig. 2a, b). Similarly, we observed a progressive increase in the prevalence of patients with liver HBV DNA >2.282 IU/cell or serum miR-181b level >2.688, reflecting increased HAI (Fig. 2c) and fibrosis scores (Fig. 2d).

Discussion

Considerable research attention has focused on the identification of novel serum miRNAs for diagnosis of diseases, in view of their noninvasive nature [25–27]. The discovery of serum miRNA in peripheral blood has provided a novel approach for noninvasive clinical diagnosis. Zhang et al. [28] evaluated circulating miR-122 in plasma of patients and animal models with viral-, alcohol-, and chemical-induced liver diseases, and suggested its potential as a liver injury biomarker for diagnosis and monitoring of disease. Another recent study showed that serum miR-29 is correlated with disease progression in CHB patients [20], supporting the potential of serum miRNAs as markers for disease progression.

Recent studies have identified cellular and viral miRNAs as a new class of regulators in viral pathogenesis [29]. Chronic HBV infection is a known major etiological factor for HCC development [1]. HBV gene expression and replication may be affected by several miRNAs that bind directly to viral transcripts, such as miR-151-5p [30, 31], miR-125-5p [31], miR-199-3p and miR-210 [32]. For example, Liu et al. [33] reported that hsa-miR-125-5p is increased at least 3-fold in the HBV-producing cell line, HepG2.2.15, compared with the parent cell line, HepG2. Potenza and colleagues previously reported that overexpression of miR-125-5p in cultured PLC/PRF/5 induces

Fig. 1 Serum miR-181b levels in CHB patients and correlation with liver HBV DNA levels. **a** Serum miR-181b levels in healthy controls (*n* = 72) and patients with CHB (*n* = 64). **b** Serum miR-181b levels in CHB patients from HBsAg (+) or HBsAg (−) groups. **c** Serum miR-181b levels in CHB patients with liver HBV DNA ≤ 2.282 or > 2.282. *Vertical lines* indicate the range, and *horizontal boundaries* of the *boxes* represent the first and third quartiles. *P < 0.0001. **d** Correlation between serum miR-181b and liver HBV DNA levels in patients with CHB. Significance was calculated according to Spearman correlation. *P* and *r* values are shown
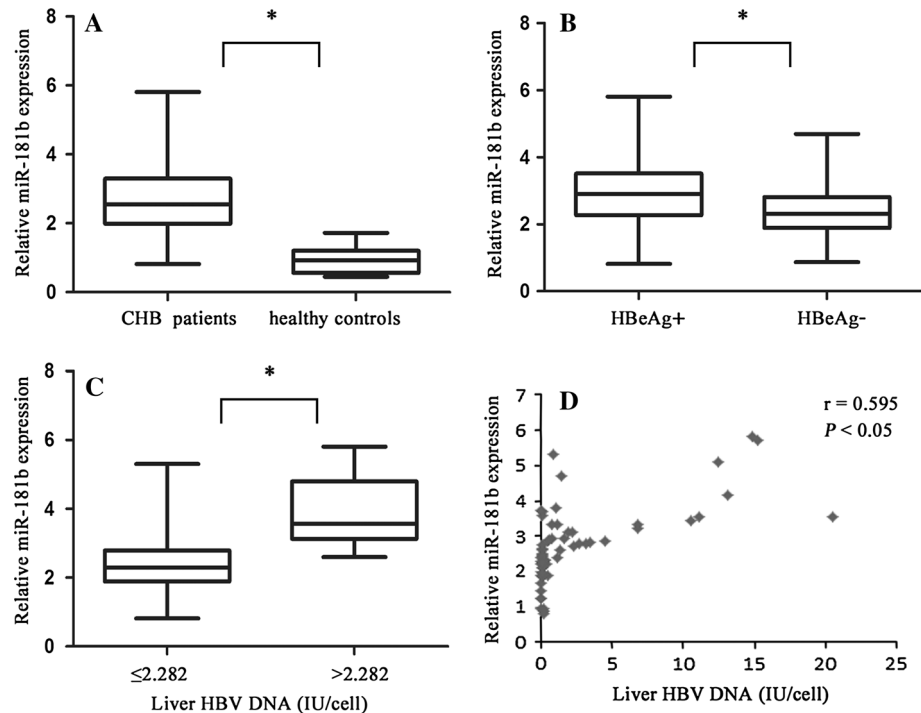

Table 2 Serum miR-181b and liver HBV DNA levels according to demographic, initial biochemical, virological and histological data

Parameter	Number	Serum miR-181b median (range)	P value[a]	Liver HBV DNA (IU/cell) median (range)	P value[a]
Age					
≤40 years	31	2.650 (0.820–5.810)	0.871	0.546 (0.006–14.870)	0.194
>40 years	33	2.410 (0.970–4.700)		0.716 (0.011–20.540)	
Sex					
Male	27	2.210 (0.820–5.810)	0.223	0.375 (0.002–20.540)	0.279
Female	37	2.650 (1.240–4.700)		0.425 (0.014–13.160)	
ALT (U/L)					
≤35	38	2.490 (0.820–5.810)	0.237	0.113 (0.006–2.140)	0.004
>35	26	2.625 (1.870–5.300)		2.485 (0.002–20.540)	
S-HBV DNA					
≤10E3IU/mL	31	2.210 (0.820–5.810)	0.017	0.135 (0.002–2.310)	0.034
>10E3IU/mL	33	2.770 (1.890–4.700)		0.716 (0.014–20.540)	
HAI score					
≤8	49	2.420 (0.820–4.700)	0.001	1.056 (0.002–20.540)	0.830
>8	15	3.450 (1.890–5.810)		1.310 (0.006–11.140)	
Fibrosis score					
≤4	39	2.220 (0.820–4.700)	<0.0001	0.098 (0.002–20.540)	0.006
>4	25	3.320 (1.890–5.810)		1.100 (0.006–14.870)	

S-HBV DNA serum HBV DNA, *HAI* histological activity index

[a] Wilcoxon rank test

significant reduction in HBsAg, whereas inhibition of miR-125-5p triggers HBsAg expression [31]. Further studies confirmed that liver miR-125-5p activity in HBV-infected tissues is correlated with HBV replication and disease progression [24]. However, since it is difficult to obtain liver tissue, liver miRNAs do not effectively serve as potential biomarkers for CHB patients. On the other hand, blood-derived microRNAs are easily accessible and can be utilized as novel markers for assessment of disease severity. In patients with chronic viral hepatitis, elevated levels

Table 3 Logistic regression analysis for independent predictors of HAI >8 and fibrosis score >4 (Ishak)

Parameters	Odd ratios	95 % CI		P values
		Lower	Upper	
HAI >8				
Age	1.071	0.980	1.171	0.131
Serum miR-181b	4.172	1.838	9.473	0.009
Liver HBV DNA	1.064	0.909	1.245	0.442
Fibrosis score >4				
Age	1.075	0.992	1.166	0.077
Serum miR-181b	5.387	2.067	14.036	0.001
Liver HBV DNA	1.010	0.876	1.163	0.895

of microRNAs, such as miR-122, have been reported [34]. In the current study, we observed miR-181b expression in the serum of all enrolled patients.

We focused on miR-181b owing to its pro-fibrosis ability [19], as well as the earlier finding that serum levels may be a potential diagnostic biomarker for cirrhosis [11].

In the current study, serum miR-181b levels were determined in CHB patients according to several bioclinical and histological parameters. Compared with healthy controls, serum miR-181b was increased in CHB patients, supporting its utility as a biomarker of CHB. Further

analysis of the correlation between serum miR-181b levels and viral replication revealed that serum miR-181b is increased in patients with HBeAg+ and HBV DNA levels higher than 2.282, suggesting that liver exposure to HBV induces serum miR-181b. Serum miR-181b was significantly correlated with serum and liver HBV DNA levels (Fig. 1d; Table 2). However, the issue of whether miR-181b is capable of inhibiting HBV replication requires further investigation. Additionally, serum miR-181b level was associated with HAI score >8 and fibrosis score >4, highlighting its potential as an independent predictor of moderate and severe CHB disease. This may be explained by the fact that higher serum miR-181b levels are induced at higher levels of HBV DNA, which is often associated with more severe liver disease [35]. These results are in accordance with our previous in vitro findings [19], suggesting that miR181b acts as pro-fibrosis miRNA in liver.

Our results support the development of serum miR-181b as a marker for disease progression in CHB patients. However, the current study has a number of limitations. The sample size was relatively small. In addition, the relationship between serum miR-181b and immune status of patients was not examined. For example, subjects were not divided into inactive carriers, low replicative HBe antigen-negative elevated ALT, high replicative HBe antigen-negative normal ALT, high replicative HBe antigen-

Fig. 2 Serum miR-181b levels and liver HBV DNA levels were analyzed according to HAI and fibrosis scores. Serum miR-181b levels according to HAI score (**a**) and fibrosis score (**b**). Liver HBV DNA levels according to HAI score (**c**) and fibrosis score (**d**)
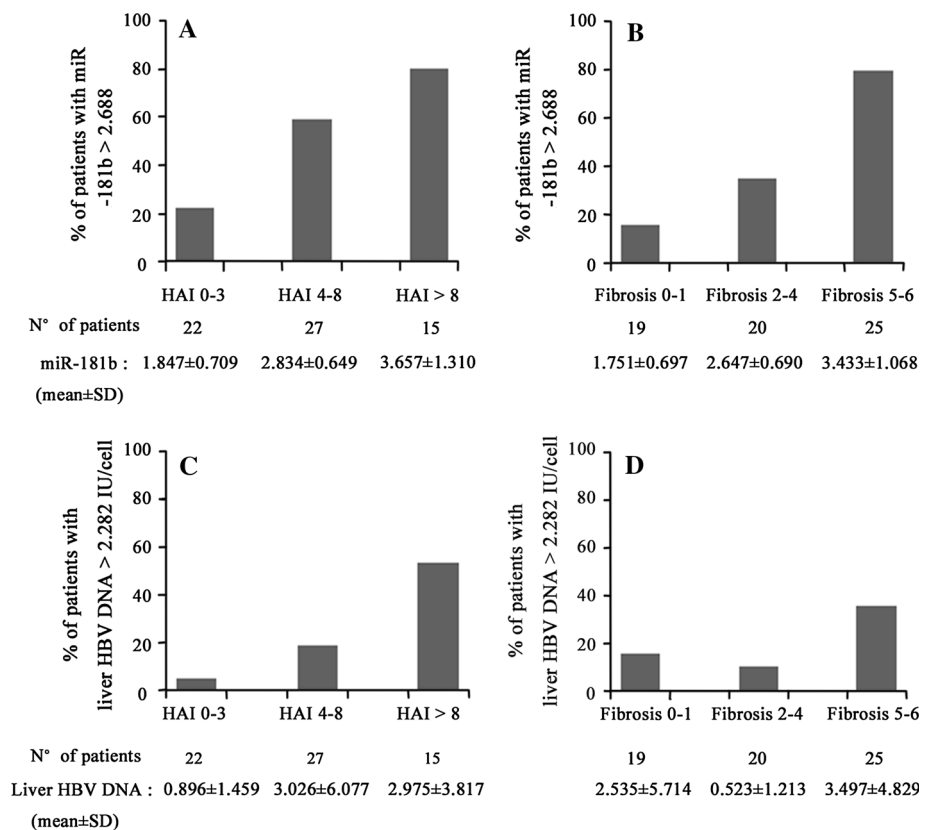
negative elevated ALT, and HBe antigen-positive hepatitis groups for analysis [34]. Studies with larger sample sizes are warranted to validate the efficacy of this marker and determine the relationship between serum miR-181b and patient immune status.

In summary, serum 181b levels are elevated in CHB patients and correlated with HBV replication and disease progression, highlighting the potential utility of this microRNA as a marker of CHB.

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