

Plasma microRNA: A novel non-invasive biomarker for HBV-associated liver fibrosis staging

TIE-ZHENG WANG¹, DONG-DONG LIN¹, BO-XUN JIN¹, XIANG-YING SUN² and NING LI¹

¹Department of General Surgery, Beijing YouAn Hospital, Capital Medical University, Beijing 100069;

²Beijing QuantoBio Biotechnology Co. Ltd., Beijing Economic-Technological Development Area, Beijing 100176, P.R. China

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Abstract. The aim of the present study was to evaluate the potential use of 7 plasma miRNAs for liver fibrosis staging in patients with chronic hepatitis B virus (HBV) infection. Relative levels of miRNAs were measured using quantitative polymerase chain reaction and used to develop a diagnostic panel. A receiver operating characteristic (ROC) curve was drawn to evaluate the performance of individual miRNAs and the whole panel. It was identified that hsa-miR-122 exhibited significantly different expression levels between F4 and F3, F2, F1, and F0 fibrosis stages ($P<0.05$), and between F2 and F1 stages ($P=0.045$); hsa-miR-146a-5p, hsa-miR-29c-3p and hsa-miR-223 exhibited significantly different expression levels between F4 and F0 stages. ROC analysis revealed that hsa-miR-122-5p, hsa-miR-223 and hsa-miR-29c-3p identified patients with \geq F2 fibrosis with area under the curve (AUC) = 0.745, 0.631 and 0.670, respectively. hsa-miR-122-5p identified patients with \geq F3 disease (AUC=0.783). hsa-miR-122-5p, hsa-miR-223 and hsa-miR-29c-3p identified patients with cirrhosis with AUC=0.776, 0.617 and 0.619, respectively. The miRNA panel exhibited a higher accuracy compared with individual miRNAs in discriminating between \geq F2, \geq F3 and F4 fibrosis stages with AUC=0.904, 0.889 and 0.835, respectively. hsa-miR-122-5p, hsa-miR-146a, hsa-miR-29c and hsa-miR-223 were positively correlated with fibrosis stage. hsa-miR-122-5p and hsa-miR-381-3p were negatively correlated with alanine aminotransferase, aspartate transaminase and HBV viral DNA load. These 7 miRNAs may serve as potential biomarkers of liver fibrosis in patients with HBV-associated fibrosis. The miRNA panel may serve as a novel non-invasive method for liver fibrosis staging.

Introduction

According to the Chinese Society of Hepatology, the most common cause of liver fibrosis is chronic viral hepatitis B (CHB) infection (1). Globally, 30% of cirrhosis cases are caused by hepatitis B virus (HBV) (2). Cirrhosis is the most advanced stage of liver fibrosis, which may develop into hepatocellular carcinoma (HCC). Liver cirrhosis is considered to be the final pathological stage of fibrosis, and fibrosis is the precursor of cirrhosis (3).

The early detection of fibrosis would provide additional time for drug treatment, which may reverse fibrosis. At present, liver biopsy is considered the 'gold standard' for identifying liver fibrosis. However, this procedure is invasive, painful and incurs the risk of complications (4); non-invasive diagnostic methods would resolve these issues. However, current, non-invasive procedures are costly and not readily accessible in developing countries, including China (5). There is an urgent requirement for a precise predictive biomarker for liver fibrosis.

MicroRNAs (miRNAs) are a series of short noncoding regulatory RNA molecules that contain 20-25 nucleotides, bind to the 3'-untranslated region of target miRNAs, and interfere with the output of a number of protein-coding genes at the post-translational level by inducing translational arrest, which in turn decreases or prevents protein synthesis (6,7). It has been established that they serve critical roles in plant and animal development, cell lineage differentiation, virus-host interactions, oncogenesis and immune responses (7). Plant miRNAs introduced through diet have been identified in the sera and tissues of various animals, which indicates that exogenous plant miRNAs may modulate the expression of target genes in mammals. Previously, miRNAs were identified to act in cross-kingdom manner (i.e., the same miRNAs were identified to be active in a number of different species and kingdoms), which is an important function (8). miRNAs affect gene expression primarily through epigenetic mechanisms and additionally regulate the epigenetic network in different pathological conditions and diseases (9). miRNAs are stable in plasma and may serve as critical diagnostic biomarkers and in disease therapy development. In addition, the number of studies examining the association between the expression of miRNAs and liver fibrosis has increased, which has highlighted the role of miRNAs in the development of liver fibrosis staging tools.

Correspondence to: Professor Ning Li or Professor Dong-Dong Lin, Department of General Surgery, Beijing YouAn Hospital, Capital Medical University, 8 Xitoutiao, Beijing 100069, P.R. China
E-mail: liningbjyah@vip.sina.com
E-mail: ldd1231@sohu.com

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Patients and methods

Patient selection. All patients were selected based on medical and pathological data. In addition, all patients were positive for HBsAg and did not have any other liver diseases, including cholangiocarcinoma, hepatitis C virus (HCV) infection, or alcoholic, autoimmune or metabolic liver diseases. Written informed consent was obtained from each participant, and the study was approved by the Ethics Review Committee of Beijing YouAn Hospital (Beijing, China). The study was conducted in accordance with the principles of the Declaration of Helsinki and the standards of Good Clinical Practice (as defined by the International Conference on Harmonization) (10).

Liver histology. Specimens were collected from patients with HBV through two methods: Ultrasound-guided percutaneous liver biopsy or wedge biopsy during an open splenectomy and esophagogastric devascularisation (hereafter referred to as surgery). Meta-analysis of Histological Data in Viral Hepatitis (METAVIR) scoring was applied to determine the severity of the fibrosis (11). F0 refers to no fibrosis, and F4 refers to liver cirrhosis. This procedure to determine which METAVIR stage each specimen was in was performed by two pathologists.

Noninvasive assessment of liver fibrosis. The aspartate aminotransferase-to-platelet ratio index (APRI) and fibrosis-4 (FIB-4) scores were derived from routine blood markers, and calculated as follows: APRI score, $APRI = [aspartate\ transaminase\ (AST) \times (upper\ limit\ of\ normal\ of\ AST)] \times 100 / platelet\ count\ (PLT)$ (12); FIB-4 score, $FIB-4 = (age\ of\ patient \times AST) / PLT \times alanine\ aminotransferase\ (ALT^{1/2})$ (13). The liver stiffness measurement (LSM) was calculated using FibroScan® 502 transient elasticity imager (Echosens, Paris, France). For the LSM, patients were placed in a supine position and the right arm was lifted to fully expose the intercostal space. The 7th, 8th and 9th intercostal spaces of the right axillary line were selected for the continuous detection of LSM. Data were represented as the median value of ≥ 10 valid shots. A reliable result was defined as ≥ 10 valid shots, a success rate of $\geq 60\%$ and an interquartile range $< 30\%$ of the median LSM value (14).

Serum ALT and AST activity levels. Blood specimens were collected using a BD Vacutainer Plus (BD Biosciences, Franklin Lakes, NJ, USA) and separated from serum by centrifugation at $3,000 \times g$ for 5 min at room temperature. The separated serum was aliquoted and stored at $-80^{\circ}C$. Serum ALT and AST activity levels were determined on the ADVIA 2400 Clinical Chemistry System using ALT (cat. no. 74046) and AST (cat. no. 74045) reagents (all Siemens AG, Munich, Germany). These contemporary methods for measurement of enzyme activity are all standardized to International Federation of Clinical Chemistry reference measurement procedures (15,16).

RNA isolation and quantification. In the present study, 7 candidate miRNAs were selected: *Homo sapiens* (Hsa)-miR-122-5p, hsa-miR-21-5p, hsa-miR-146a-5p, hsa-miR-29c-3p, hsa-miR-223, hsa-miR-22-3p and hsa-miR-381-3p based on a previous study by our group (17).

Human plasma samples were collected in Biomedical Information Center at Beijing YouAn Hospital. Venous blood samples were collected using a BD Vacutainer Plus and centrifuged at $700 \times g$ at room temperature for 15 min within 4 h of collection. The plasma supernatant was immediately recovered and stored at $-80^{\circ}C$. RNA was isolated using QuantoBio Total RNA Isolation Kit (Beijing QuantoBio Biotechnology Co., Ltd., Beijing, China), then sample quantity and quality were evaluated with a Nanodrop™ 8000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). All samples successfully passed quality control [RNA purity: (optical density) $OD_{260}/OD_{280} = 1.8-2.1$; RNA concentration: $\geq 1.0\ ng/\mu l$] for miRNA isolation and were screened using a QuantoBio three-step quantitative polymerase chain reaction (qPCR) method. Quanto EC1 and EC2 (Beijing QuantoBio Biotechnology Co., Ltd.) were introduced as external controls during RNA isolation, polyadenylation, cDNA synthesis and qPCR. Reverse transcription was started using *Escherichia coli* polyA polymerase (New England Biolabs, Ipswich, MA, USA) to add a polyA tail at the 3' end of RNAs. Then, with RT primer annealing, a universal tag was attached to the 3' end of cDNAs during the cDNA synthesis using a M-MLV reverse transcriptase (Beijing QuantoBio Biotechnology Co., Ltd.) for 1 h at $42^{\circ}C$. The whole polyA product was added into the $40\ \mu l$ RT solution, which included 500 U M-MLV with 1X RT buffer, 0.5 mM dNTP and $20\ \mu M$ RT primer (Table I). qPCR was performed with SYBR® Premix Ex Taq™ II (TaKaRa) and the primers in Table I. Universal Primers Mix, which target the universal tag in RT primer, were the down stream primers used in qPCR for every miRNA. Upstream primer were specific to the miRNA. The thermocycling conditions were as follows: Initial denaturation at $95^{\circ}C$ for 5 min, followed by 40 cycles of $95^{\circ}C$ for 30 sec and $60^{\circ}C$ for 1 min. The dissociation stage was as follows: $95^{\circ}C$ for 15 sec, $60^{\circ}C$ for 1 min and $95^{\circ}C$ for 15 sec. The data were normalized using the external controls Quanto EC1 and Quanto EC2 and analysed with a RealTime StatMiner (Integromics, Granada, Spain). The relative expression level of miRNA was calculated using the $2^{-\Delta\Delta C_q}$ method (18).

HBV load measurements. Human blood samples were collected in Biomedical Information Center at Beijing YouAn Hospital. Venous blood samples were collected using a BD Vacutainer Plus and centrifuged at $1,000 \times g$ at room temperature for 20 min within 24 h of collection. DNA was isolated from plasma using the QIAamp DNA Mini kit (Qiagen, Inc., Valencia, CA, USA) and stored at $-20^{\circ}C$ prior to use. The qPCR reactions were performed using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The $20\ \mu l$ qPCR mixture comprised of 250 nmol/l 2X SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, Inc.), 900 nmol/l HBV-specific sense primers (5'-CTCTCTTTA CGCGGTCTC-3'), HBV-specific antisense primers (5'-GTC GTTGACATTGCTGAG-3'), 250 nmol/l HBV probe (5'-CCG TCTGTGCCTTCTCATCTGC-3') and $4\ \mu l$ DNA sample. The thermocycling conditions were as follows: Initial denaturation at $95^{\circ}C$ for 10 min, followed by 45 cycles of denaturation at $94^{\circ}C$ for 30 sec, annealing and extension step at $57^{\circ}C$ for 60 sec. The final extension step at $98^{\circ}C$ for 10 min, then continued at $4^{\circ}C$

Table I. Primers used in the quantitative polymerase chain reaction analysis.

Name	Sequence (5'-3')	Target miRNA
RT	AAGCAGTGGTATCAACGCAGAGTGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	Universal
FP1	CAAGGGCAAGCTCTCTG	hsa-miR-381-3P
FP2	GAGTTCTTCAGTGGCAAGC	hsa-miR-22-3p
FP3	GAGAACTGAATTCCATGGGT	hsa-miR-146a-5p
FP4	GAACGCCATTATCACACT	hsa-miR-122-5p
FP5	GCTTATCAGACTGATGTTG	hsa-miR-21-5p
FP6	GCACCATTGAAATCGGT	hsa-miR-29c-3p
FP7	TGTCAGTTTGTCAAATACC	hsa-miR-223
FP-EC1	CAACCTCCTAGAAAGAGTA	cel-miR-67-3p
FP-EC2	TGAGCAACGCGAACAATCA	cel-miR-356a
UPM-L	CTCACACGACTCACGACAGGGCAAGCAGTGGTATCAACGCAGAGTG	Universal
UPM-S	CTCACACGACTCACGACAGGGC	Universal

hsa, *Homo sapiens*; cel, *Caenorhabditis elegans*; miR, microRNA; RT, reverse transcription; FP, forward primer; EC, external control; UPM, Universal Primers MicroRNA; L, long; S, short.

indefinitely. Cq values were generated and linear regression analyses of the calibration curves were performed by CFX™ Manager Software 3.1 (Bio-Rad Laboratories, Inc.). The data generated by the qPCR was normalized according to the guidelines of the World Health Organization International Standard for Hepatitis B Virus DNA Nucleic Acid Amplification Techniques (19). HBV DNA levels are expressed in the units, IU/ml.

Statistical analysis. All data were analysed using SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean \pm standard deviation (SD), number (percentage) or median (25-75% percentiles) when appropriate. Categorical data were compared by χ^2 or Fischer's exact test when appropriate. Clinical data from the five independent fibrosis groups (F0-F4) were normally distributed and analysed using a one-way analysis of variance followed by a Least Significant Difference test. HBV load were measured using qPCR and log-transformed for parametric statistical tests. miRNA expression levels from independent samples from two fibrosis groups (\geq F2 vs. F0-F1; \geq F3 vs. F0-F2; F4 vs. F0-F3) were compared by Mann-Whitney U-test, and multiple groups, including the 5 fibrosis subgroups, were compared by Kruskal-Wallis test and a Dunn-Bonferroni test for post-hoc comparisons. The diagnostic accuracy of plasma miRNAs was examined using the area under the curve (AUC) of corresponding receiver operating characteristic (ROC) curves. Univariate and multivariate logistic regression analyses were performed to identify predictor miRNAs associated with the risk of \geq F2, \geq F3 or F4. The predicted probability according to the panel was used as a diagnostic marker to construct the ROC curve. Spearman correlation analysis was used to examine the association between clinical parameters including the LSM, FIB-4 and APRI scores, serum ALT and AST activity levels, hepatitis B virus (HBV) expression, and miRNA expression. MedCalc (version 12.7; MedCalc Software bvba, Ostend, Belgium)

was used to calculate and compare the AUCs and assess the diagnostic values of the miRNAs of interest. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Description and clinicopathologic features of patients. A total of 92 patients (26 females and 66 males), who provided liver tissue and plasma specimens in Beijing YouAn Hospital affiliated with Capital Medical University from June 2014 to October 2016, were enrolled in the present study. The mean age of these patients was 40.58 ± 11.43 years (range, 16-60 years). The mean body mass index (BMI) was 22.87 ± 3.04 kg/m², and the Model for end-stage liver disease (MELD) index was 5.25 ± 4.40 . Patients were divided into five subgroups by liver histology result: F0; F1; F2; F3; and F4. Among these patients, 11 patients exhibited F0 stage, 16 patients exhibited F1 stage, 12 patients exhibited F2 stage, 13 patients exhibited F3 stage, and 40 patients exhibited F4 stage disease. The characteristics of the participants are presented in Table II. LSM, FIB-4, serum ALT, and HBV DNA levels were significantly different among the subgroups ($P < 0.05$). There was no significant difference in the distribution of age, sex, MELD, Child-Pugh score (20), APRI, BMI or AST among the five groups ($P > 0.05$).

Relative expression of the 7 selected miRNAs at different fibrosis stages. Plasma miRNAs were analysed using the Cq value by qPCR. The threshold levels were set as follows: miRNA, Cq < 35 ; detection rate $> 75\%$. Then, these values were transformed using the $2^{-\Delta\Delta Cq}$ method (18). Kruskal-Wallis and a Dunn-Bonferroni test for post-hoc comparisons was used to compare 5 subgroups (Fig. 1). It was identified that hsa-miR-122 exhibited a significantly different expression between F4 and F3, F2, F1 and F0 stages ($P = 0.012$, 0.045, 0.001 and 0.028, respectively; Fig. 1A) and between F2 and F1 stages ($P = 0.032$; Fig. 1A). hsa-miR-21-5p expression was not identified to be significant between groups (Fig. 1B). hsa-miR-146a-5p exhibited significantly different expression

Table II. Characteristics of participants by fibrosis stage.

Characteristics	Fibrosis stage					P-value
	F0	F1	F2	F3	F4	
No. of cases (%)	11 (12)	16 (17.4)	12 (13)	13 (14.1)	40 (43.5)	
Sex						0.481
Male, n (%)	9 (9.8)	13 (14.1)	8 (8.7)	7 (7.6)	29 (31.5)	
Female, n (%)	2 (2.2)	3 (3.3)	4 (4.3)	6 (6.5)	11 (12.0)	
Age, years	34.7±12.8	34.8±13.0	40.2±10.1	38.0±10.2	45.5±9.3 ^{a-d}	0.192
Child-Pugh score	5.3±0.7	5.6±1.4	5.5±1.0	5.8±0.6	6.1±1.2 ^a	0.228
MELD	3.5±5.0	4.5±5.6	3.7±2.9	4.4±3.5	6.8±4.0 ^{a,c}	0.055
BMI	22.8±4.0	24.0±2.4	23.8±3.8	22.2±3.9	22.7±2.7	0.783
LSM, KPa	5.9±2.0	7.8±4.4	9.4±3.5	14.0±9.1	22.8±14.1 ^{a-d}	<0.0001
HBV DNA, log IU/ml	5.2±2.5	4.9±2.4	3.9±2.0	4.5±2.2 ^a	3.0±1.6 ^{a-d}	0.003
ALT, U/l	32.1±16.8	185.7±222.0 ^{a,d,e}	105.7±145.3	83.62±89.0	37.7±34.3	0.001
AST, U/l	26.6±13.6	101.1±107.5	50.3±46.6	158.1±379.2 ^{a,e}	42.9±35.9	0.121
FIB-4	0.8±0.4	1.8±1.6	3.0±4.6	3.0±2.7	6.1±6.2 ^{a-d}	0.002
APRI	0.3±0.1 ^d	1.6±1.6	1.0±0.9	3.4±7.6	1.9±1.9	0.192
Method of obtaining liver tissue samples						<0.0001
Liver biopsy, n	11	15	10	9	4	
OSED, n	0	1	2	4	36	

Data are presented as mean ± standard deviation. ^aP<0.05 vs. F0; ^bP<0.05 vs. F1; ^cP<0.05 vs. F2; ^dP<0.05 vs. F3; ^eP<0.05 vs. F4. MELD, Model for end-stage liver disease; BMI, Body mass index; LSM, Liver stiffness measurement; ALT, alanine aminotransferase; AST, aspartate transaminase; APRI, AST to platelet ratio index; HBV, hepatitis B virus; FIB-4, fibrosis-4; OSED, open splenectomy and esophagogastric devascularization.

levels between F4 and F0 stages (P=0.037; Fig. 1C), and hsa-miR-29c-3p exhibited significantly different expression levels between F0 and F2 stages (P=0.034), and between F0 and F4 stages (P=0.007; Fig. 1D). hsa-miR-223 exhibited significantly different expression between F4 and F0 stages (P=0.017; Fig. 1E). hsa-miR-22-3p and hsa-miR-381-3p expression was not identified to be significant between groups (Fig. 1F and G).

Diagnostic potential of individual plasma miRNAs. To evaluate whether the 7 selected plasma miRNAs were able to be used as potential diagnostic markers for liver fibrosis, ROC curves were generated to evaluate their performance. The ROC curves revealed that several plasma miRNAs significantly identified ≥F2, ≥F3 and F4 stages (P<0.05; Fig. 2). hsa-miR-122-5p, hsa-miR-223 and hsa-miR-29c-3p identified patients with ≥F2 stage with AUC=0.745 (0.643-0.830), 0.631 (0.524-0.730) and 0.670 (0.564-0.765), respectively. hsa-miR-122-5p identified patients with ≥F3 stage disease with AUC=0.783 (0.685-0.862). hsa-miR-122-5p, hsa-miR-223 and hsa-miR-29c-3p were able to significantly identify patients with cirrhosis (F4 stage) with AUC=0.776 (0.677-0.856), 0.617 (0.510-0.717), and 0.619 (0.512-0.719), respectively. The 95% confidence intervals (CI), sensitivities, specificities, positive predictive values (PPV) and negative predictive values (NPV) for the miRNAs that identified patients with ≥F2, ≥F3, and F4 stage disease are summarized in Table III.

Establishment of a predictive and non-invasive diagnostic panel with selected plasma miRNAs. A logistic regression model was applied to predict the probability of diagnosing different fibrosis statuses using miRNA diagnostic markers. The panel, which included these 7 miRNAs, was identified to be a significant predictor for the risk of ≥F3 diagnosis and was used to construct the ROC curve. The predicted probability of diagnosing ≥F3 stage disease was calculated from the logit model, and the ROC curve was drawn using the following equation: Logit (P)=0.912-5.696 x hsa-miR-122-5p+0.060 x hsa-miR-21-5p+6.444 x hsa-miR-146a-5p-6.234 x hsa-miR-29c-3p-1.955 x hsa-miR-223+10.789 x hsa-miR-22-3p-45.954 x hsa-miR-381-3p.

Diagnostic performance of the predictive panel. The ROC analysis demonstrated that the complete panel exhibited increased accuracy in discriminating between severe fibrosis (≥F3) and patients with no/mild/moderate (F0-F2) fibrosis compared with individual miRNAs, with AUC=0.889 (95% CI, 0.806-0.945; P<0.0001), sensitivity=83.02%, specificity=87.18%, PPV=89.8% and NPV=79.1%. In addition, it also exhibited improved performance in discriminating between patients with significant fibrosis (≥F2) from patients with no/mild fibrosis (F0-F1) compared with individual miRNAs, with AUC=0.904 (95% CI, 0.825-0.956; P<0.0001), sensitivity=81.54%, specificity=92.59%, PPV=96.4% and NPV=67.6%. The model was also able to significantly

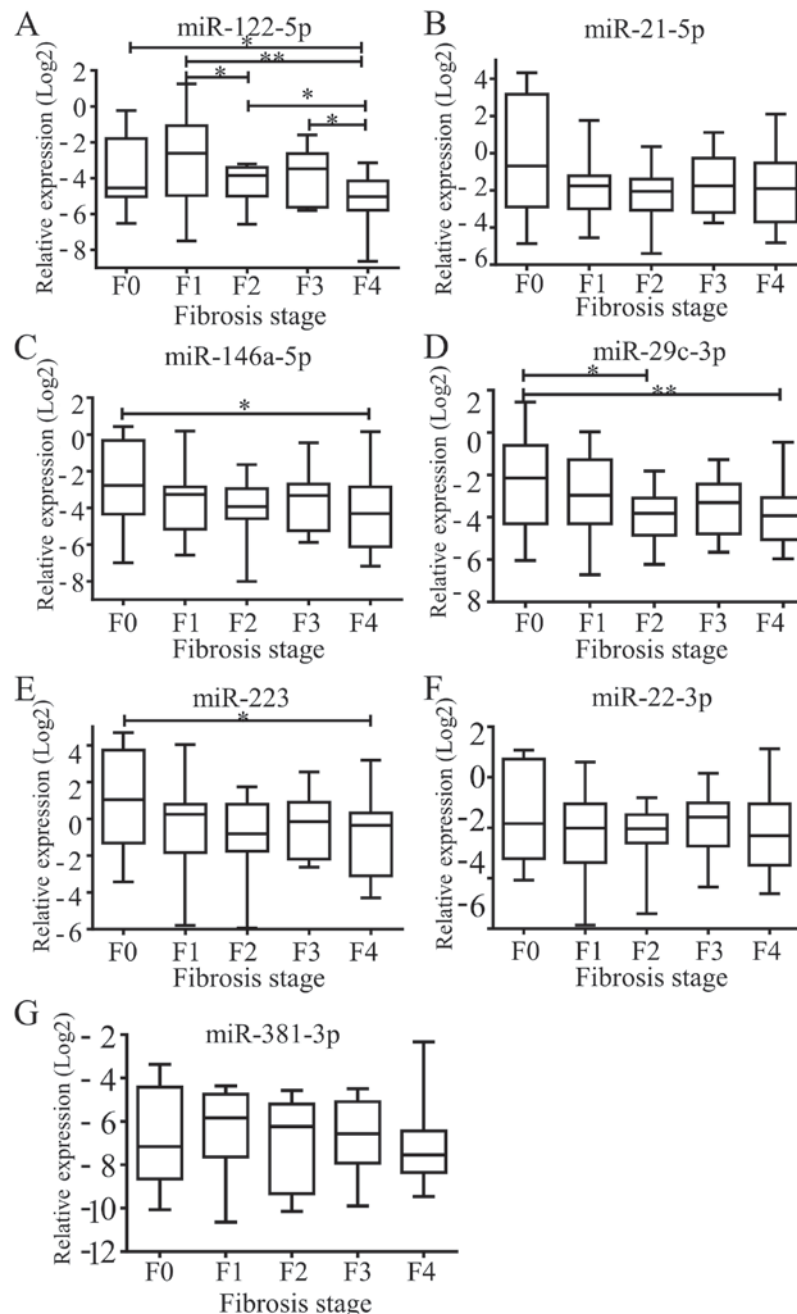


Figure 1. Relative expression of selected miRNAs in association with fibrosis stage. All miRs are human (i.e., hsa-miR). Relative expression levels of (A) miR-122-5p, (B) miR-21-5p, (C) miR-146a-5p, (D) miR-29c-3p, (E) miR-223, (F) miR-22-3p and (G) miR-381-3p in different fibrosis stages are presented. The levels of miR-122-5p, miR-146a-5p, miR-29c-3p and miR-223 in different fibrosis stages reached statistical significance and were analysed using a Kruskal-Wallis and Dunn-Bonferroni test. * $P<0.05$ and ** $P<0.01$. miR, microRNA.

identify patients with F4 disease from patients at other fibrosis stages with $AUC=0.835$ (95% CI, 0.743-0.904; $P<0.0001$), sensitivity=87.50%; specificity=73.08%, PPV=71.4%, and NPV (certainty to exclude F4)=88.4% (Fig. 2D).

Correlation of plasma miRNA expression with fibrosis, LSM, FIB-4, APRI and serum biochemical values. It was identified that LSM, FIB-4 and APRI exhibited positive correlations with fibrosis stage ($r=0.701$, $P<0.001$; $r=0.661$, $P<0.001$; and $r=0.416$, $P<0.001$, respectively; Fig. 3A), and hsa-miR-122-5p, hsa-miR-146a, hsa-miR-29c and hsa-miR-223 were identified to be positively correlated with fibrosis stage ($r=0.409$, $P<0.001$; $r=0.212$, $P=0.043$; $r=0.252$, $P=0.015$; and $r=0.232$, $P=0.026$, respectively) (Fig. 3B).

A total of 6 miRNAs (hsa-miR-122-5p, hsa-miR-146a-5p, hsa-miR-29c-3p, hsa-miR-223, hsa-miR-22-3p and hsa-miR-381-3p) exhibited a positive correlation with LSM ($r=0.350$, $P=0.003$; $r=0.290$, $P=0.014$; $r=0.342$, $P=0.004$; $r=0.274$, $P=0.021$; $r=0.260$, $P=0.028$; and $r=0.304$, $P=0.010$, respectively; Fig. 3C). A total of 6 miRNAs (hsa-miR-122-5p, hsa-miR-21-5p, hsa-miR-146a-5p, hsa-miR-29c-3p, hsa-miR-223 and hsa-miR-381-3p) were positively correlated with FIB-4 ($r=0.419$, $P<0.001$; $r=0.272$, $P=0.009$; $r=0.376$, $P<0.001$; $r=0.360$, $P<0.001$; $r=0.409$, $P<0.001$; and $r=0.238$, $P=0.023$, respectively; Fig. 4A) and 5 miRNAs (hsa-miR-21-5p, hsa-miR-146a-5p, hsa-miR-29c-3p, hsa-miR-223 and hsa-miR-22-3p) were positively correlated with APRI

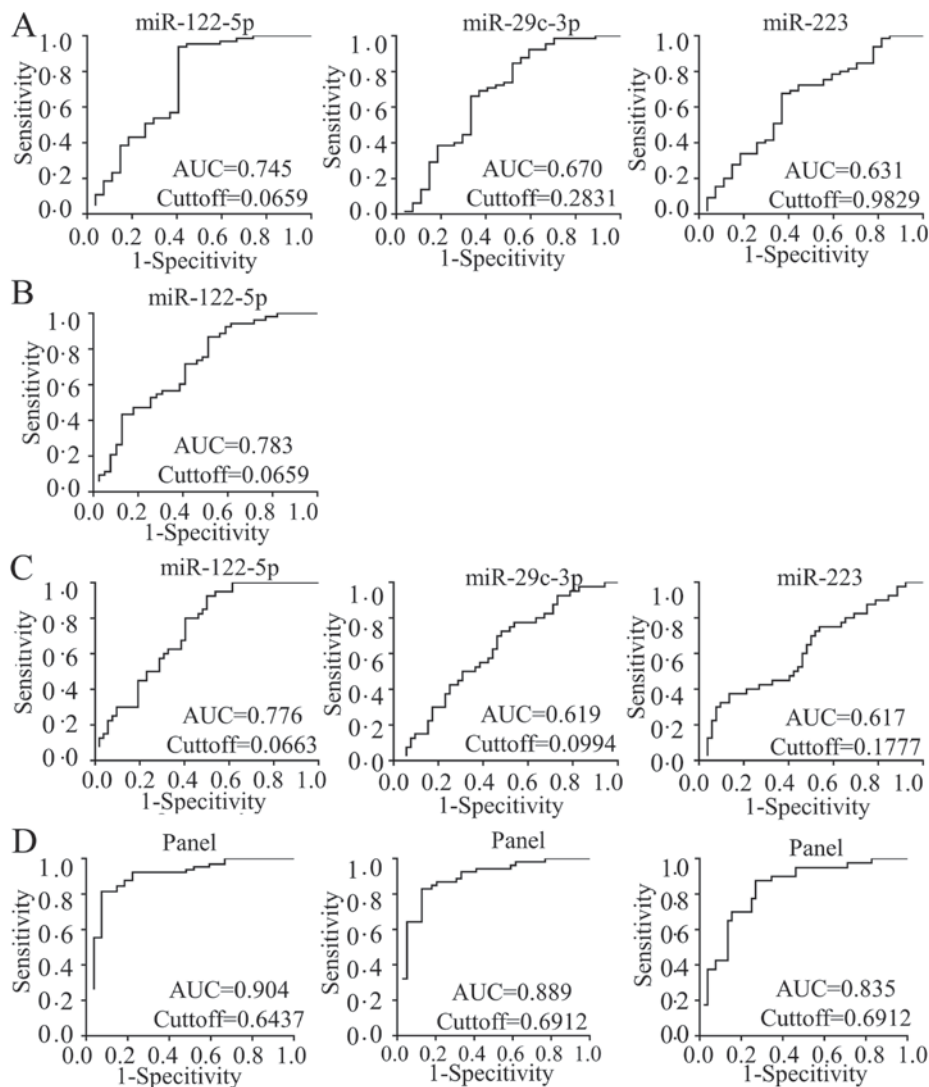


Figure 2. Receiver operating characteristic curve analysis of individual miRNAs and the complete panel. All miRs are human (i.e., hsa-miR). (A) miR-122-5p, miR-29c-3p and miR-223 were identified to be able to discriminate significant fibrosis (\geq F2) from no/mild fibrosis (F0-F1). (B) miR-122-5p was able to discriminate between severe fibrosis (\geq F3) and no/mild/moderate fibrosis (F0-F2). (C) miR-122-5p, miR-29c-3p and miR-223 were able to distinguish cirrhosis (F4) from no/any fibrosis (F0-F3) stages. (D) The performance of the miRNA panel for discriminating between significant fibrosis (\geq F2; left), severe fibrosis (\geq F3; middle) and cirrhosis (F4; right). miR, microRNA; AUC, area under the curve.

($r=0.277$, $P=0.008$; $r=0.363$, $P<0.001$; $r=0.225$, $P=0.031$; $r=0.414$, $P<0.001$; and $r=0.212$, $P=0.043$, respectively; Fig. 4B). A total of 3 miRNAs (hsa-miR-122-5p, hsa-miR-29c-3p and hsa-miR-381-3p) were negatively correlated with HBV load ($r=-0.514$, $P=0.000$; $r=-0.332$, $P=0.001$; and $r=-0.437$, $P<0.001$, respectively; Fig. 4C). hsa-miR-122-5p ($r=-0.458$, $P<0.001$) and hsa-miR-381-3p ($r=-0.242$, $P=0.021$) were negatively correlated with ALT activity (Fig. 4D) and AST levels (hsa-miR-122-5p, $r=-0.328$, $P=0.001$; and hsa-miR-381-3p, $r=-0.239$, $P=0.022$; Fig. 4E).

Discussion

The pathophysiological mechanism of hepatic fibrosis is a disruption between the production and dissolution of the extracellular matrix triggered by chronic and inflammatory injury (21). There are several other factors that cause liver disease, including viruses, bacteria, parasites, other infectious factors and non-infectious factors, including alcohol, drugs,

autoimmune disease, metabolic syndromes, genetic defects and congenital dysplasia. HBV is the most common cause of liver fibrosis in China (1). A previous study hypothesized that the clinical pathway of the majority of HBV-associated HCC follows four stages: Healthy; hepatitis; cirrhosis; and HCC (22). With the gradual improved understanding of liver fibrosis and the prevalence of treatment options, we hypothesized that the hepatic fibrosis stage should be included in this classification of clinicopathological progression. The advantage is that the natural course of HBV infection may be more accurately studied, but it does require sufficient pathological examination or LSM, APRI and FIB-4 analysis. It was demonstrated that altered miRNA patterns following chronic liver disease markedly affect the progression of fibrosis by potentially targeting the expression of extracellular matrix proteins and the synthesis of mediators of profibrogenic pathways (23). The pathological changes in liver fibrosis, which may be caused by different pathogens, may have a combination of commonalities and individual characteristics. Therefore, HBV-associated

Table III. Performance of individual plasma miRNAs.

Disease stage vs. control	miRNA	Area under the curve	95% Confidence interval	Cut-off value	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %
≥F2	miR-122-5p	0.745	0.643-0.830	0.066	72.3	74.1	87.0	52.6
	miR-223	0.631	0.524-0.730	0.983	67.7	63.0	81.5	44.7
	miR-29c-3p	0.670	0.564-0.765	0.283	92.3	40.7	78.9	68.8
≥F3	miR-122-5p	0.783	0.685-0.862	0.066	79.2	69.2	77.8	71.1
F4	miR-122-5p	0.776	0.677-0.856	0.066	90.0	61.5	64.3	88.9
	miR-223	0.617	0.510-0.717	0.178	37.5	86.5	68.2	64.3
	miR-29c-3p	0.619	0.512-0.719	0.099	72.5	51.9	53.7	71.1

miRNA, microRNA.

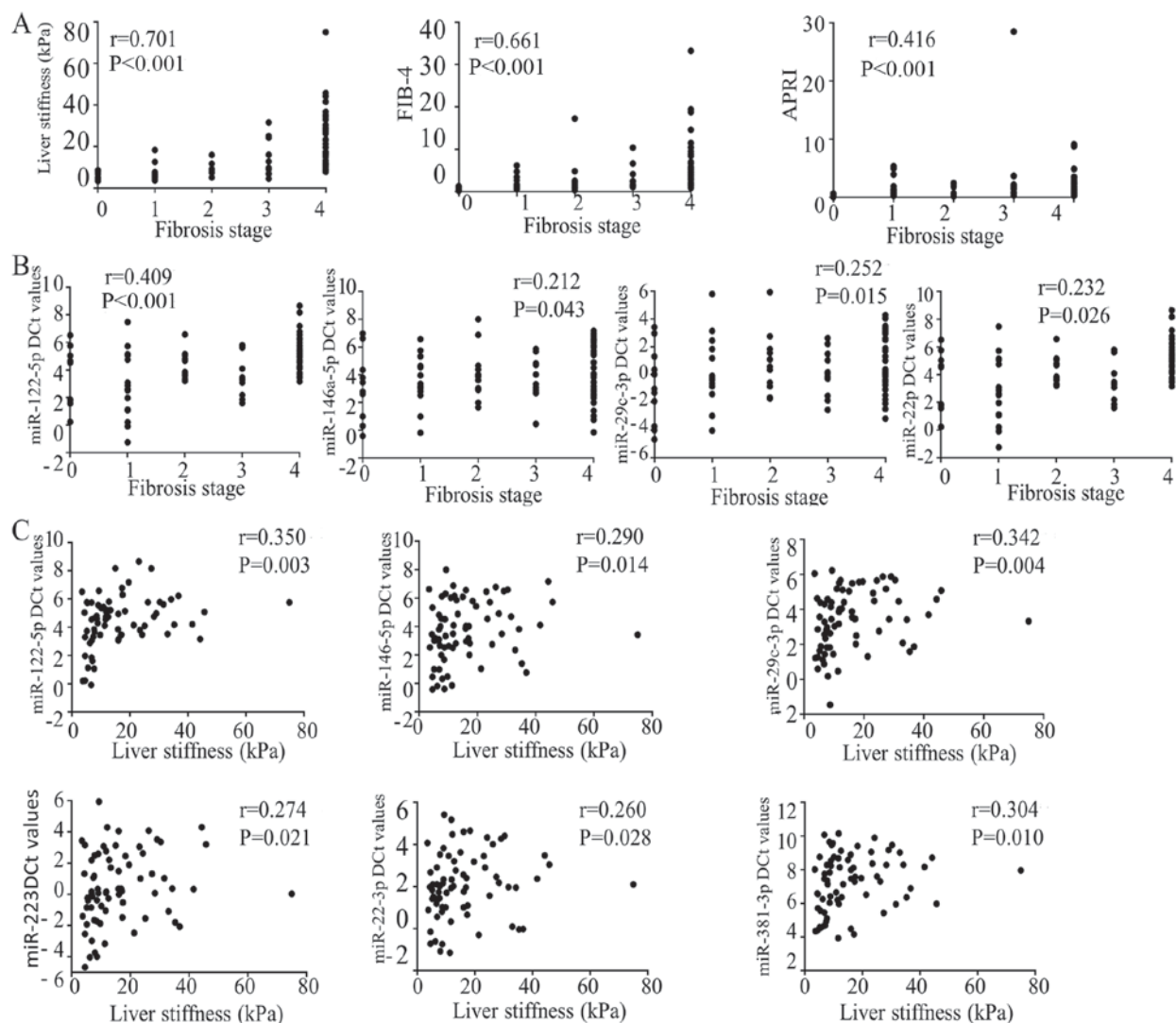


Figure 3. Correlations between plasma miRNA expression and LSM and fibrosis stage. All miRs are human (i.e., hsa-miR). (A) LSM, FIB-4 and APRI exhibited positive correlations with fibrosis stage. (B) miR-122-5p, miR-146a-5p, miR-29c-3p and miR-223 were identified to be positively correlated with fibrosis stage. (C) A total of 6 miRNAs (miR-122-5p, miR-146a-5p, miR-29c-3p, miR-223, miR-22-3p and miR-381-3p) demonstrated positive correlations with LSM. miR, microRNA; FIB-4, fibrosis-4; APRI, aspartate transaminase to platelet ratio index; LSM, Liver stiffness measurement.

liver fibrosis patients were intentionally enrolled in the present study to remove the potential interference of other factors.

Cirrhosis is the most advanced stage of fibrosis, which may develop into HCC. Therefore, the diagnosis and staging of

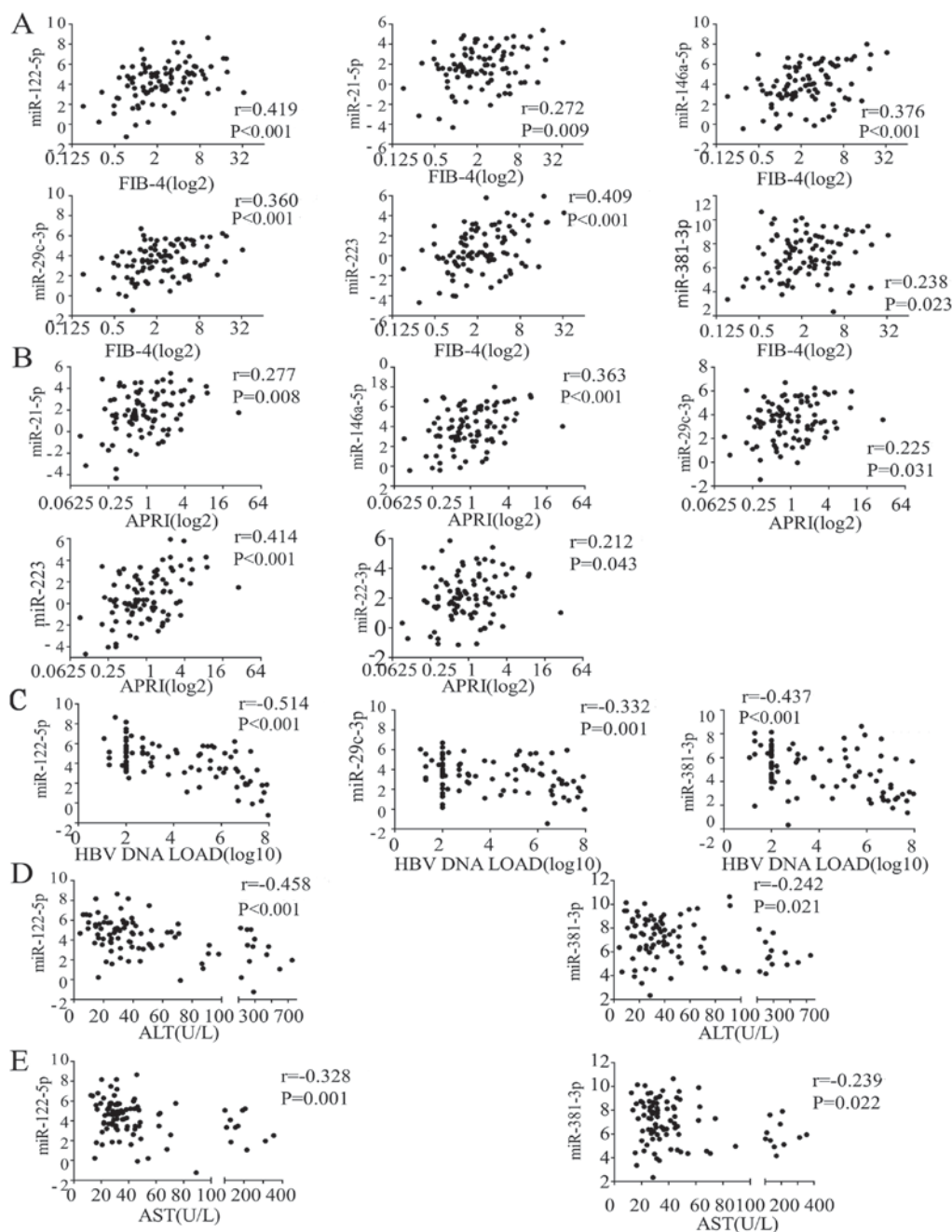


Figure 4. Correlations between plasma miRNA expression levels and hepatic function and viral load. All miRs are human (i.e., hsa-miR). (A) A total of 6 miRNAs (miR-122-5p, miR-21-5p, miR-146a-5p, miR-29c-3p, miR-223 and miR-381-3p) were positively correlated with FIB-4. (B) A total of 5 miRNAs (miR-21-5p, miR-146a-5p, miR-29c-3p, miR-223 and miR-22-3p) were positively correlated with APRI. (C) A total of 3 miRNAs (miR-122-5p, miR-29c-3p, and miR-381-3p) were negatively correlated with HBV DNA load levels. miR-122-5p and miR-381-3p were negatively correlated with (D) ALT and (E) AST activity levels. miR, microRNA; FIB-4, fibrosis-4; HBV, hepatitis B virus; ALT, alanine aminotransferase; AST, aspartate transaminase; APRI, AST to platelet ratio index.

HBV-associated liver fibrosis in patients is important and may guide the appropriate timing of antiviral therapy. In the present study, it was identified that the miRNA expression data of several patients with clinically-diagnosed CHB and cirrhosis were not consistent with their pathological results, therefore patients who were diagnosed with liver cirrhosis did not have pathological results that verified the diagnosis. As the liver has a strong compensatory capacity (24); consequently certain patients with liver cirrhosis may have mild or asymptomatic clinical manifestations. Concomitantly, specific cases exhibited clinically-diagnosed cirrhosis with minor pathological changes. In the present study, the pathological results

of 7 cases (16.2%) (1 case of F1, 2 cases of F2, and 4 cases of F3) did not reach an early diagnosis of liver cirrhosis (F4). The reason may be that the wedge biopsies during surgery were not representative of the actual clinical state. Although liver biopsy remains the 'gold standard' for the diagnosis of liver fibrosis, it has limitations; its invasive nature and sampling errors (i.e., the most severely fibrotic may not be obtained) have not yet been overcome. Therefore, the present study aimed to investigate the potential of circulating miRNAs as a method of diagnosis.

Circulating miRNAs, as diagnostic markers for different stages of hepatic fibrosis, have been extensively studied

in previous years (6,25-27). miRNAs are derived from blood or body fluids, making them easily accessible as potential biomarkers for the evaluation of hepatic fibrosis. Lee *et al* (28) identified the first miRNA, termed lin-4, from *Caenorhabditis elegans* in 1993. Previous evidence has suggested that there is a complex association between miRNAs and HBV genes (29-31). The mechanism of HBV infection in regulating miRNAs may be through either RNA interference or the inhibition of translational initiation and elongation (23,32-34). In addition, deregulated expression of miRNAs may be present for a long period prior to the onset of disease (35).

In the present study, it was identified that hsa-miR-122, hsa-miR-146a-5p, hsa-miR-29c-3p and hsa-miR-223 exhibited significantly different expression levels between the F4 and F0 stage, which suggests that these 4 miRNAs may participate in the progression of liver cirrhosis from CHB infection, and that they may be used as diagnostic biomarkers. Furthermore, the expression levels of hsa-miR-122 were significantly different between F4 and F3, F2 and F1 disease stages ($P=0.012$, 0.045 and 0.001 , respectively), demonstrating that it may be used as a precise fibrosis staging biomarker. These data are consistent with certain studies investigating HCV-associated diseases (36,37), non-alcoholic fatty liver disease (NAFLD) (38,39) and HCC. hsa-miR-122 is specifically and most abundantly expressed in the liver, accounting for >70% of the total miRNAs in hepatocytes (40), which may reflect the fact that the accumulation of fibrosis may be not only associated with the expression level of hsa-miR-122, but also with the duration of HBV infection. The downregulation of miR-122 between F2 from F1 ($P=0.032$), which had been previously supported by studies involving HCV and NAFLD (36,37,39), maybe a sensitive signal of hepatic injury. The mechanism may involve the positive regulation of the accumulation of cholesterol and triglycerides and the metabolism of fatty acids (39). hsa-miR-122 has been identified to suppress the proliferation of hematopoietic stem cells (HSCs), resulting in the decreased maturation of collagen by downregulating the expression of prolyl 4-hydroxylase subunit alpha 1, a key enzyme in collagen maturation (41). In addition, the expression level of plasma hsa-miR-122 may predict the survival of patients with liver cirrhosis (42). Following liver injury and fibrosis, levels of plasma hsa-miR-122 were markedly decreased depending on the severity of fibrosis (43). These data indicate that hsa-miR-122 has other unknown functions in the viral life cycle. Previous data have revealed that hsa-miR-146a promotes viral replication and deregulates the metabolic pathways associated with liver disease (32). It has also been demonstrated that hsa-miR-146a is downregulated in liver fibrotic tissues and suppresses the activation and proliferation of HSCs in the progression of fibrosis (44,45). The mechanism regulates transforming growth factor- β signalling by targeting mothers against decapentaplegic homolog 4 (46). hsa-miR-29 is also involved in the pathogenesis of fibrotic diseases, including liver cirrhosis: It was identified that hsa-miR-29 levels were significantly decreased in advanced liver cirrhosis compared with healthy controls (33). In addition, a low expression level of hsa-miR-29 was identified to be correlated with the severity of hepatic fibrosis (23). The deregulation of hsa-miR-223 was described in HBV-positive HCC and HBV/HCV-positive cirrhosis (47). In the present

study, the expression level of hsa-miR-223 was detected to be downregulated in early hepatic cirrhosis (F4) compared with CHB infection (F0). We hypothesized that deregulated levels of hsa-miR-223 are present throughout the entire process of HBV infection, and are associated with infection, inflammation and cancer, which is supported by previous studies (48,49).

There have been a number of studies examining potential biomarkers for CHB infection, liver cirrhosis and HCC (17,50). However, miRNAs for liver fibrosis staging have not been well-studied. In the METAVIR fibrosis-scoring system, F0 indicates no fibrosis, and F4 indicates liver cirrhosis. Within this system, there are several different classifications of liver fibrosis stages. For example, F0/1 represents no significant liver fibrosis, F2/3 represents progressive fibrosis and F4 represents cirrhosis. The present study explored the diagnostic performance of 7 fibrosis-associated miRNAs in identifying no/mild fibrosis (F0-F1), significant fibrosis ($\geq F2$), severe fibrosis ($\geq F3$) and cirrhosis (F4) based on the METAVIR fibrosis-scoring system. hsa-miR-122-5p, hsa-miR-223 and hsa-miR-29c-3p identified $\geq F2$ (AUC=0.745, 0.631 and 0.670, respectively) and F4 (AUC=0.776, 0.617 and 0.619, respectively). hsa-miR-223 and hsa-miR-29c-3p exhibited good predictive potential in the diagnosis of significant fibrosis or cirrhosis. In addition, hsa-miR-122-5p was also able to identify patients with $\geq F3$ disease with AUC=0.783. As a staging biomarker, the authors of the current study hypothesis that hsa-miR-122-5p may participate in the whole process of liver fibrosis and may be used to determine fibrosis status.

Although ROC analysis revealed that differentially-expressed plasma miRNAs were able to significantly identify $\geq F2$, $\geq F3$, and F4 disease ($P<0.05$), the performance of individual plasma miRNAs was not as satisfactory (AUC=0.617-0.783). Multivariate logistic analysis revealed that the panel combining the 7 miRNAs exhibited a high diagnostic accuracy for $\geq F3$ (AUC=0.889) and F4 (AUC=0.904) disease stages. However, the panel had modest results in identifying $\geq F2$ disease (AUC=0.835). The miRNA panel significantly improved the diagnostic efficacy of fibrosis compared with the diagnostic efficacy of a single miRNA, which may provide a strategy for using miRNAs to study the staging of HBV-associated liver fibrosis. In fact, a large number of studies have used miRNA panels for the diagnosis of HCC (22,50). Additional studies are required to confirm this panel and validate its significance in liver fibrosis of other aetiologies.

In the present study, the correlation between plasma miRNAs levels and disease progression in patients with CHB infection was also investigated. Plasma hsa-miR-122-5p, hsa-miR-146a, hsa-miR-29c and hsa-miR-223 levels were identified to be positively correlated with fibrosis stage, suggesting that these 4 miRNAs were involved in the progression of HBV-associated liver fibrosis. hsa-miR-122-5p and hsa-miR-381-3p were negatively correlated with ALT, AST and HBV viral DNA load. The data are presented as ΔCq values and not as $\Delta\Delta Cq$ values. These results indicated that increased levels of hsa-miR-122-5p and hsa-miR-381-3p are accompanied by HBV replication and high activity levels of ALT and AST in cultured cells. Plasma hsa-miR-122 levels were increased with the severity of liver injury, indicating a good correlation with aminotransferase levels

and necroinflammation (51,52). A correlation between hsa-miR-21 expression and viral load, fibrosis and plasma liver transaminase levels, as described in the literature (36), was not identified in the present study. In the present study, it was not determined whether hsa-miR-21 induction was a causative factor in fibrosis. However, a previous study revealed that increased hsa-miR-21 levels were associated with fibroblast dysfunction and fibrosis outcome (53). In a number of other organs, hsa-miR-21 is one of the most important miRNAs that is abundantly expressed following fibrosis induction (54-56).

In the present study, three non-invasive parameters, LSM, APRI and FIB-4, were thoroughly examined. Although they were identified to be positively associated with liver fibrosis stage, they lacked inter-stage specificity and were unable to detect the early stages of fibrosis. The majority of the miRNAs included in the present study demonstrated significant positive correlations with these non-invasive techniques, indicating that there is an established association between miRNAs and these non-invasive examination markers.

In conclusion, the present study revealed that plasma miRNAs are potential circulating markers for fibrosis and early cirrhosis diagnosis. An optimized miRNAs panel may improve diagnostic accuracy and decrease the necessity for clinical liver punctures.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

D-DL and NL conceived and designed the study. T-ZW, B-XJ and X-YS performed the experiments. T-ZW wrote the paper. T-ZW, D-DL and NL reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Written informed consent was obtained from each participant, and the study was approved by the Ethics Review Committee of Beijing YouAn Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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