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Serum MicroRNA Levels as a Noninvasive Diagnostic Biomarker for the Early Diagnosis of Hepatitis B Virus-Related Liver Fibrosis

Suxia Bao, Jianming Zheng, Ning Li, Chong Huang, Mingquan Chen, Qi Cheng, Kangkang Yu, Shengshen Chen, Mengqi Zhu, and Guangfeng Shi

Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai, China

Background/Aims: To investigate the role of selected serum microRNA (miRNA) levels as potential noninvasive biomarkers for differentiating S0-S2 (early fibrosis) from S3-S4 (late fibrosis) in patients with a chronic hepatitis B virus (HBV) infection. **Methods:** One hundred twenty-three treatment-naive patients with a chronic HBV infection who underwent a liver biopsy were enrolled in this study. The levels of selected miRNAs were measured using a real-time quantitative polymerase chain reaction assay. A logistic regression analysis was performed to assess factors associated with fibrosis progression. Receiver operating characteristic (ROC) curve and discriminant analyses validated these the ability of these predicted variables to discriminate S0-S2 from S3-S4. **Results:** Serum miR-29, miR-143, miR-223, miR-21, and miR-374 levels were significantly downregulated as fibrosis progressed from S0-S2 to S3-S4 ($p<0.05$), but not miR-16. The multivariate logistic regression analysis identified a panel of three miRNAs and platelets that were associated with a high diagnostic accuracy in discriminating S0-S2 from S3-S4, with an area under the curve of 0.936. **Conclusions:** The levels of the studied miRNAs, with the exception of miR-16, varied with fibrosis progression. A panel was identified that was capable of discriminating S0-S2 from S3-S4, indicating that serum miRNA levels could serve as a potential noninvasive biomarker of fibrosis progression. **(Gut Liver 2017;11:860-869)**

Key Words: Biomarkers; Hepatitis B virus; Hepatitis B, chronic; Liver fibrosis; MicroRNAs

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is one of the most life-threatening diseases affecting humans, as it may lead to hepatic cirrhosis, hepatocellular carcinoma, or even death. Hepatic fibrosis is one of the common characteristics of chronic liver disease. Early diagnosis and sustained follow-up of the progression of hepatic fibrosis is essential in terms of preventing hepatic cirrhosis and end-stage hepatic disease. Liver biopsy is the widely used procedure for the accurate determination of fibrosis. However, it is an invasive procedure associated with certain outcomes and has severe limitations, such as possibility of serious complications, contradictions, sampling, and intra- and inter-observer errors. Therefore, there is a general need to find indicators at the molecular level to help predict disease progression.

MicroRNAs (miRNAs) are small noncoding RNA comprising 21 to 25 nucleotides that control the expression of target genes at the posttranscriptional level by binding to the noncoding region of the target gene.[1] Several miRNAs are involved in the development of fibrosis of the lung, liver, kidney, and cardiovascular diseases.[2-5] MiR-29b is capable of suppressing hepatic stellate cell (HSC) activation, production of type I collagen,[6-8] and expression of extracellular matrix genes in HSCs through the transforming growth factor β (TGF-β)/SMAD-CTGF signaling network.[9,10] MiR-21 may regulate TGF-β2,[11] and has been shown to activate HSCs through the PTEN/AKT signaling or ERK1 signaling pathway.[12,13] Moreover, TGF-β stimulates processing of the primary miR-21 precursor into mature miR-21,[14] and miR-21 inhibition reduces liver fibrosis and prevents tumor development.[15] MiR-143 could regulate TGF-β/SMAD signaling to mediate the expression of collagen type III in stromal fibroblasts of scirrhous-type gastric cancer.[16] MiR-16 promotes liver fibrosis through downregulation of hepatocyte growth factor and SMAD7.[6,17] There has been little study of the function of miR-374 and miR-223. Moreover, miRNAs with modulation

Correspondence to: Guangfeng Shi
Department of Infectious Diseases, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, China
Tel: +86-21-52889999, Fax: +86-21-62489015, E-mail: 14111220001@fudan.edu.cn
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activity are characterized by high stability when combined with proteins in the serum and plasma, and miRNAs are also released by cells into circulation,[18] providing the possibility of evaluating circulative miRNAs as biomarkers. Therefore, miRNAs derived from blood or body fluid are easily accessible potential biomarkers for the evaluation of disease severity.

However, whether circulating miRNAs can serve as potential biomarkers for liver fibrosis has not been well evaluated. We aimed, in the present study, to investigate the role of selected serum miRNAs as a potential noninvasive biomarker to differentiate S0-S2 (early fibrosis) from S3-S4 (late fibrosis) in patients with chronic HBV infection.

MATERIALS AND METHODS

1. Study subjects

A total of 123 treatment-naive patients with chronic HBV infection who were admitted to the Department of Infectious Diseases, Huashan Hospital, Fudan University (Shanghai, China) from January 2014 to August 2016 were recruited in the study. All the patients were hepatitis B surface antigen (HBsAg)-positive for at least 6 months. Exclusion criteria were (1) patients co-infected with human immunodeficiency virus; (2) those with the coexistence of liver injury caused by any other etiologies including hepatitis C virus infection, hepatitis D virus infection, drug intake, alcohol consumption, and autoimmune hepatitis, and so on; (3) those with severe systematic diseases; and (4) pregnancy and lactation. In all, 123 patients who underwent liver biopsy and 20 healthy individuals serving as healthy controls (HCs) were finally enrolled in the study. Patient characteristics are summarized in Table 1.

Written informed consent was obtained from all the adult patients who participated in the study. Patients aged below 18 years provided their verbal assent and written informed consent was obtained from their parents. The study was performed in accordance with the Helsinki Declaration and was approved by the Ethical Committee of Huashan Hospital, Fudan University.

2. Blood sampling, clinical characteristics, and laboratory examinations

Peripheral blood samples were collected at the day of liver biopsy and were centrifuged at 3,000×*g* for 10 minutes at room temperature. Then, the serum samples were aliquoted and centrifuged at 3,000×*g* for an additional 10 minutes at 4°C to remove any remaining cellular debris. The serum was immediately stored at –80°C until analysis. Liver function tests were determined by standard methods in a clinical setting. HBsAg titers were determined for stored frozen serum samples with an HBsAg quantitative assay (Abbott Laboratories, Abbott Park, IL, USA) based on the automated chemiluminescent microparticle immunoassay (Abbott Architect i2000SR analyzer; Abbott Laboratories). Samples with HBsAg >250 IU/mL were diluted to the calibration range. A domestic HBV DNA quantification assay (Shanghai Kehua Bio-engineering Co., Ltd., Shanghai, China) was used to quantify serum HBV DNA titers. Samples with HBV DNA >4×10^7 IU/mL were further diluted and retested.

3. Liver histology

Liver specimens obtained by liver biopsy using a 16-gauge Menghini needle in patients with chronic HBV infection were

Table 1. Characteristics of the Enrolled Patients with a Chronic HBV Infection and HCs at Baseline

Parameter	HBV infection (n=123)			HCs (n=20)	p-value*
	S0-S2 (n=69)	S3-S4 (n=54)	p-value		
Sex (male/female)	45/24	39/15	0.224	11/9	0.891
Age, yr	28 (26–36)	39 (31–49)	<0.001	31 (28–43)	0.912
ALT, U/L	83 (51–200)	41 (27–123)	0.001	19 (7–32)	<0.0001
AST, U/L	47 (28–88)	42 (33–83)	0.669	16 (6–28)	0.127
WBC, $\times10^9$/L	5.2 (4.5–5.9)	3.5 (2.2–5.6)	<0.001	NA	
PLT, $\times10^9$/L	195 (164–215)	78 (45–164)	<0.001	NA	
ALB	44 (40–46)	39 (38–43)	<0.001	NA	
HBsAg, $\log_{10}$ IU/mL	4.0 (3.5–4.7)	3.5 (2.9–3.9)	<0.0001	NA	
HBV DNA, $\log_{10}$ IU/mL	7.5 (5.6–7.8)	5.0 (0.0–7.1)	<0.001	NA	
HBeAg (+/–)	48/21	40/14	0.367	NA	
Grading of inflammation (G0/G1/G2/G3/G4)	14/29/17/9/0	0/16/16/18/4			
Stage of fibrosis (S0/S1/S2/S3/S4)	23/22/24/0/0	0/0/0/26/28			

Data are presented as median (interquartile range).
HBV, hepatitis B virus; HCs, healthy controls; ALT, alanine transaminase; AST, aspartate amino transferase; WBC, white blood cell; NA, not available; PLT, platelet; ALB, albumin; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.
*HBV versus HCs, p<0.05.

assessed by two experienced pathologists. The experienced clinical pathologist then examined the sections for inflammation (G) and staging of fibrosis (S) using a modified Scheuer scoring system.[19] Two components, grading (G) and staging (S), given in a numerical value ranging from 0 to 4, were used to describe disease progression of chronic HBV infection. S0 represent the absence of fibrosis; S1 represent enlarged, fibrotic portal tracts; S2 represent periportal or portal-portal septa, but intact architecture; S3 represent fibrosis with architectural distortion, but no obvious cirrhosis; whereas S4 represent definite cirrhosis.

4. RNA isolation and quantitative reverse transcription-polymerase chain reaction

Total miRNA was extracted and purified from 150 μL serum using the miRNeasy Serum/Plasma Kit (QIAGEN GmbH, Hilden, Germany). Before extraction, 3.5 μL cel-miR-39 prediluted at 1.6×10^8 copies/μL was added to each tube as a spike-in control for normalization. The purified miRNA was immediately reverse-transcribed with the miScript II Reverse Transcription Kit (QIAGEN GmbH). The expression of serum miR-29a, miR-29b, miR-29c, miR-143, miR-223, miR-21, miR-374, and miR-16 was quantified using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A real-time reaction was performed using miScript SYBR Green PCR Kit with primers specific for miR-29a, miR-29b, miR-29c, miR-143, miR-223, miR-21, miR-374, and miR-16 (all from QIAGEN GmbH) according to the manufacturer's instructions. All reactions were performed in triplicate. A comparative ΔCT method was used to compare each target with cel-miR-39, and relative values were expressed as $2^{-\Delta\Delta CT}$.

5. Statistical analysis

Values are expressed as mean±standard deviation, median (25% to 75% percentiles) or number (percentage) when appropriate. The Mann-Whitney U-test was used to determine intergroup differences. The correlation coefficients (r) were calculated using Spearman correlation. Logistic regression analysis was performed to identify predictor miRNAs and clinical parameters associated with the risk of HBV-related fibrosis progression from S0-S2 to S3-S4. Receiver operating characteristic (ROC) and the area under ROC curve (AUC) were used to calculate the diagnostic values of the studied miRNAs and clinical parameters for differentiation fibrosis progression. The cutoff value was defined by the sum of sensitivity and specificity achieving its maximum.

The discriminant analysis is a multivariate statistical method of classification, and the classification of a case is based on the combination of prior probabilities with discriminant functions. Therefore, the discriminant analysis was performed to further confirm the predictive efficiency of prediction model for fibrosis

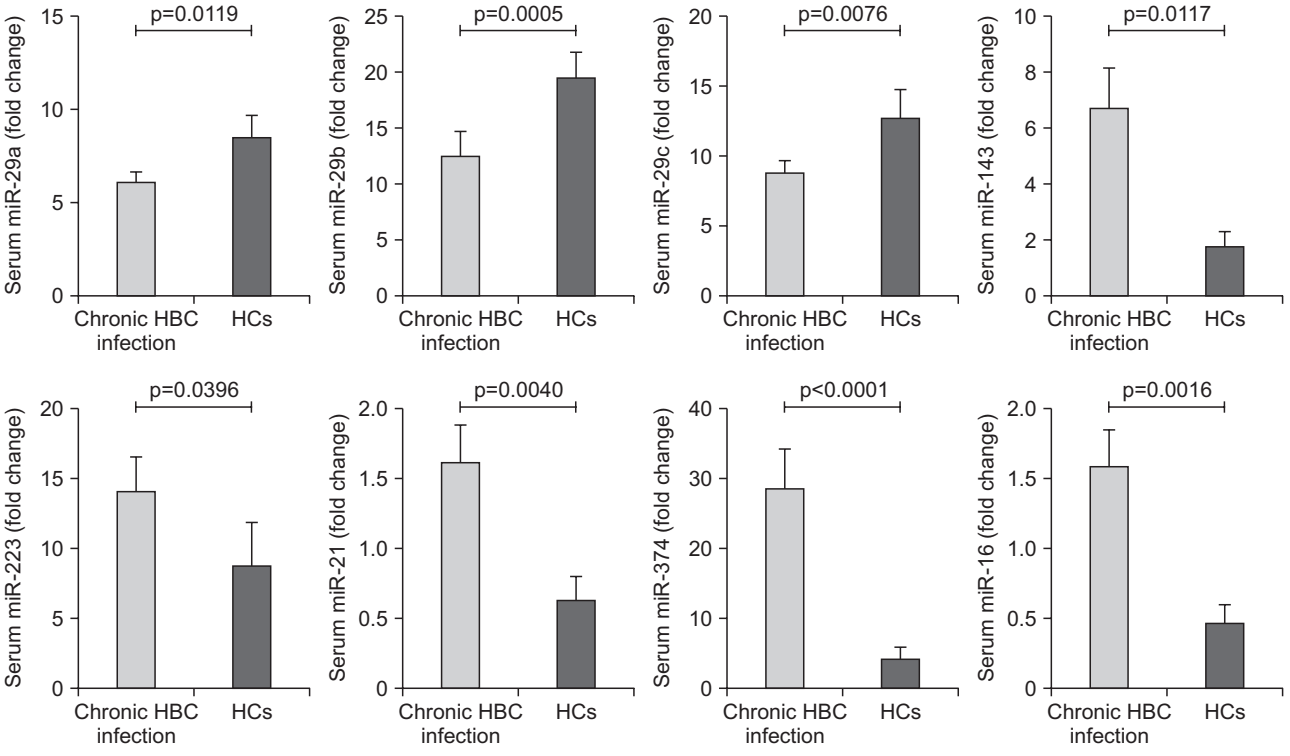


Fig. 1. Differences in serum microRNA (miRNA) levels in patients with a chronic hepatitis B virus (HBV) infection and healthy controls (HCs). The relative serum levels of miR-29a, miR-29b, miR-29c, miR-143, miR-223, miR-21, miR-374, and miR-16 in patients with a chronic HBV infection (n=123) were compared with those in HCs (n=20). Data were analyzed using the Mann-Whitney U-test. A p-value <0.05 is considered statistically significant.

progression from S0-S2 to S3-S4 in HBV-related liver fibrosis. All statistical tests were two-tailed, and a probability level of $p<0.05$ was considered as statistically significant. Data were analyzed using SPSS software for Windows version 19.0 (IBM Corp., Armonk, NY, USA).

RESULTS

1. Differential expression of serum miRNA levels during HBV-related liver disease progression

We first examined serum miRNA profiles in patients with chronic HBV infection using the Mann-Whitney U-test. Compared to HCs, serum miR-143, miR-223, miR-21, miR-374, and miR-16 were significantly upregulated in patients with chronic HBV infection (HBV vs HCs; $p<0.05$, $p<0.05$, $p<0.001$, $p<0.0001$, and $p<0.001$, respectively), whereas miR-29a, miR-29b, and miR-29c were significantly downregulated in patients with chronic HBV infection (HBV vs HCs; $p<0.05$, $p<0.0001$, and $p<0.001$, respectively) (Fig. 1). In a detailed analysis, we investigated if the studied miRNAs in patients could be deregulated during individual fibrosis stages (S0 to S4) using the nonparametric Kruskal-Wallis test. Interestingly, all the studied miRNAs except miR-16 were significantly downregulated in S3 and S4, and they were not differentially expressed in fibrosis stages S0, S1, and S2 (Fig. 2). The detailed results revealed that all the studied miRNAs were significantly downregulated in S4 compared to S0, S1, and S2 ($p<0.05$) except miR-16, which was upregulated with fibrosis progression from S0 to S4 with no statistical significance; miR-29a, mir-29c, and miR-143 were also significantly downregulated from S3 to S4 ($p<0.05$). Moreover, serum miR-29b, miR-223, and miR-374 levels were significantly downregulated in S3 compared to S0, S1, and S2 ($p<0.05$). However, the finding was not statistically significant in S0, S1, and S2 ($p>0.05$). We then examined if the studied miRNA levels changes from S0-S2 to S3-S4 or from G0-G2 to G3-G4 by using the Mann-Whitney U-test. The results showed that there were no significant differences in miRNA levels between G0-G2 and G3-G4 (Supplementary Fig. 1), whereas all the studied miRNAs were significantly downregulated during fibrosis progression from S0-S2 to S3-S4 except miR-16 ($p<0.05$) (Supplementary Fig. 2).

2. Diagnostic performance of studied miRNAs for discriminating liver fibrosis progression from S0-S2 to S3-S4

As mentioned above, studied miRNAs except miR-16 in pa-

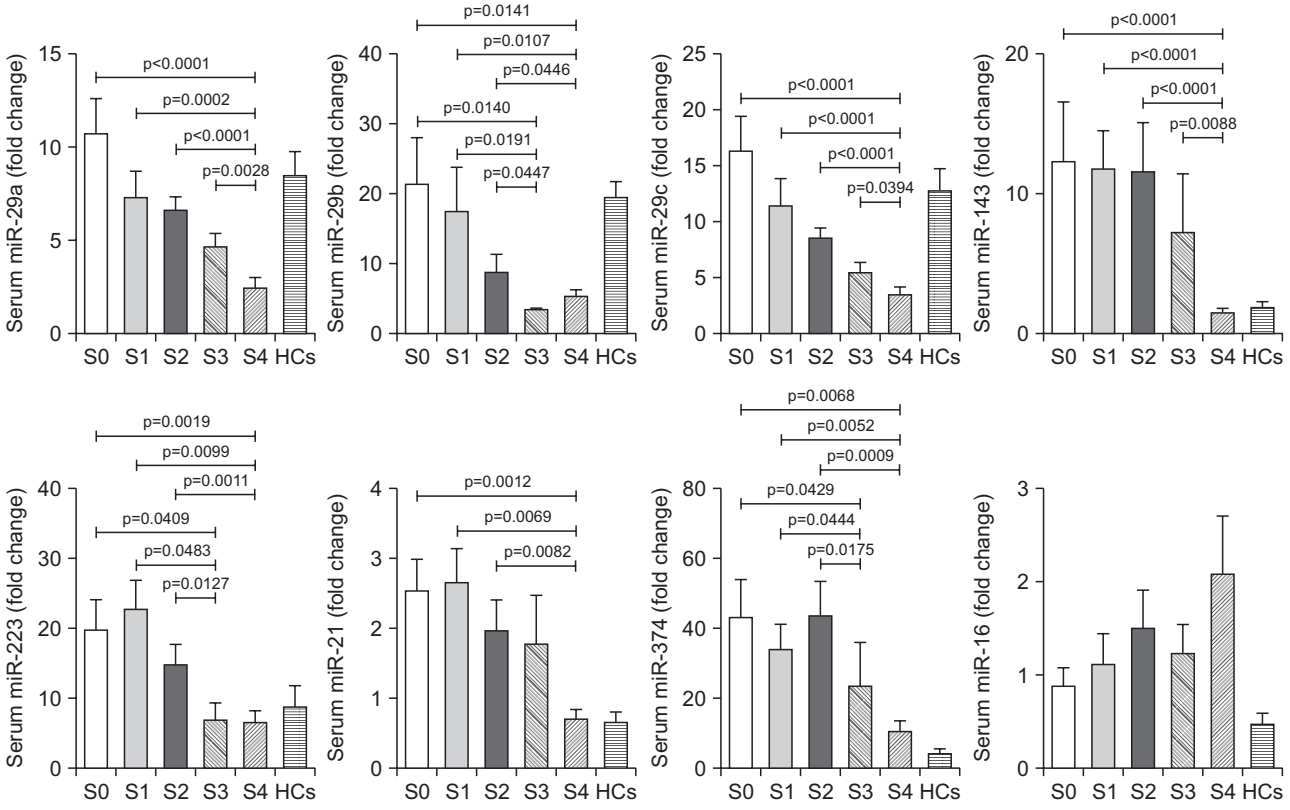


Fig. 2. Signature of relative serum microRNA (miRNA) levels during hepatitis B virus (HBV)-related liver disease progression. A detailed analysis of the relative serum levels of miR-29a, miR-29b, miR-29c, miR-143, miR-223, miR-21, miR-374, and miR-16 at different fibrosis stages (S0, n=23; S1, n=22; S2, n=24; S3, n=26; S4, n=28) in patients with a chronic HBV infection (n=123) is shown. Data were compared using the Kruskal-Wallis test.
HCs, healthy controls.

tients with chronic HBV infection significantly downregulated during individual fibrosis progression from S0-S2 to S3-S4, raising the possibility that the studied miRNAs could discriminate fibrosis progression from S0-S2 to S3-S4. Indeed, ROC analysis revealed that the studied miRNAs could discriminate S0-S2 from S3-S4 with AUC=0.8030 for miR-29a (76.36% sensitivity and 72.22% specificity), 0.7237 for miR-29b (72.73% sensitivity and 58.33% specificity), 0.8121 for miR-29c (89.09% sensitivity and 61.11% specificity), 0.8123 for miR-143 (70.97% sensitivity and 77.27% specificity), 0.7806 for miR-223 (59.46% sensitivity and 85.71% specificity), 0.7589 for miR-21 (56.25% sensitivity and 85.71% specificity), 0.7282 for miR-374 (52.00% sensitivity and 79.55% specificity), and 0.4081 for miR-16 (80.40% sensitivity and 13.90% specificity), respectively (Fig. 3). The calculated sensitivities, specificities, and cut-off value for the studied miRNAs to discriminate S0-S2 from S3-S4 in patients with chronic HBV infection are shown in Supplementary Table 1.

3. The three-miRNA and PLT panel is an independent predictive factor for discriminating liver fibrosis progression from S0-S2 to S3-S4

Univariate and multivariate logistic regression analysis of the studied miRNAs and clinical parameters such as white blood

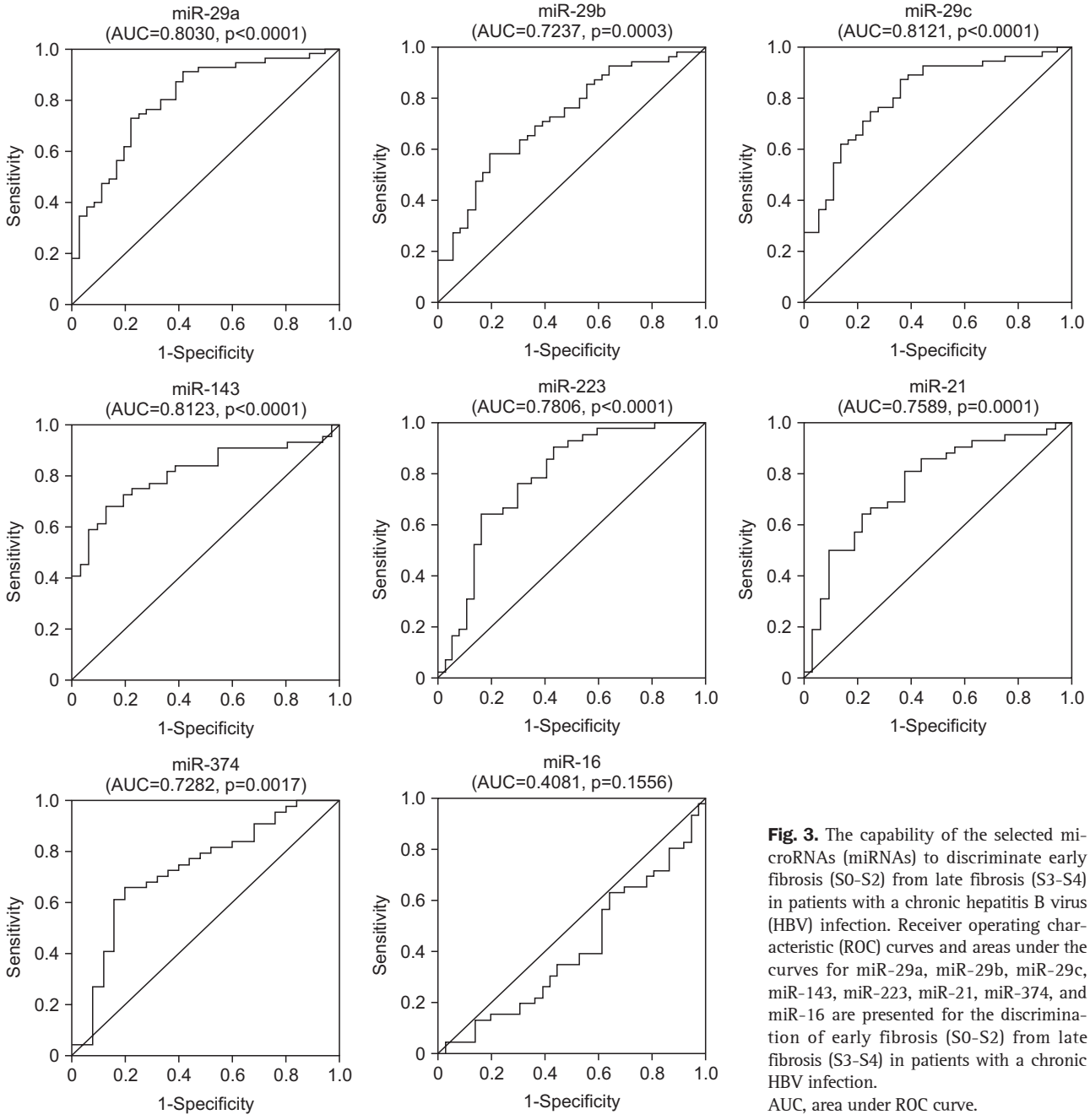


Fig. 3. The capability of the selected microRNAs (miRNAs) to discriminate early fibrosis (S0-S2) from late fibrosis (S3-S4) in patients with a chronic hepatitis B virus (HBV) infection. Receiver operating characteristic (ROC) curves and areas under the curves for miR-29a, miR-29b, miR-29c, miR-143, miR-223, miR-21, miR-374, and miR-16 are presented for the discrimination of early fibrosis (S0-S2) from late fibrosis (S3-S4) in patients with a chronic HBV infection.
AUC, area under ROC curve.

cell (WBC), platelet (PLT), albumin (ALB), HBsAg, and HBV DNA which were significantly associated with S3-S4 were performed to select the predictor parameters associated with HBV-related liver fibrosis progression from S0-S2 to S3-S4. The results revealed that all the studied miRNAs and the studied clinical parameters except miR-16 are selected as significant predictors associated with the changes in HBV-related liver cirrhosis progression from S0-S2 to S3-S4 in the univariate analysis (Table 2). Correlation analysis and variance inflation factor (VIF) among the studied miRNAs revealed that miR-29a and miR-29c were highly collinearity (r=0.967 between miR-29a and miR-29c, VIF=26.545 for miR-29a and VIF=26.925 for miR-29c) (Supplementary Table 2). Moreover, the correlation coefficient of miR-29a was higher than miR-29c in PLT, WBC and ALB (Supplementary Table 3), so we delete miR-29c for further analysis. In a stepwise forward multivariate analysis, miR-29a, miR-143, miR-223, and PLT were significant predictors of the risk of HBV-related liver fibrosis progression from S0-S2 to S3-S4. The predicted probability of having the risk of HBV-related liver fibrosis progression from S0-S2 to S3-S4 from the logit model was based on the three-miRNA and PLT panel (Table 2), Logit (P)=1.042-0.165*miR-29a-0.064*miR-143-0.491*miR-223-0.349*PLT. Similarly, miR-29a, miR-143, miR-223, and PLT were significant predictors of the risk of HBV-related liver fibrosis progression from S0-S1 to S2-S4 and from S0-S3 to S4 (data not shown).

4. The three-miRNA and PLT panel is superior to APRI and FIB-4 for discriminating liver fibrosis progression from S0-S2 to S3-S4

The diagnostic performance of the established three-miRNA and PLT panel for discriminating fibrosis progression from S0-

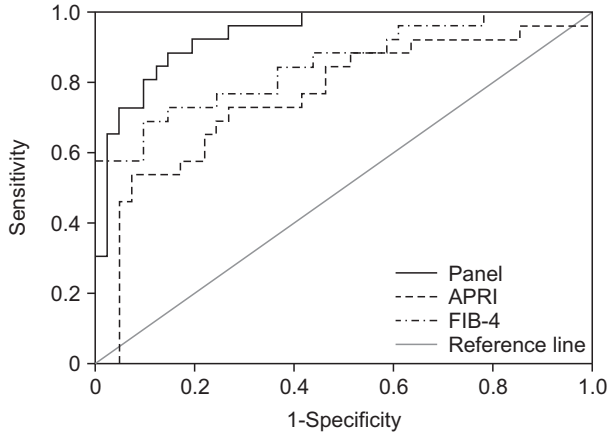
Table 2. Logistic Regression Analysis to Discriminate Early Fibrosis (S0-2) from Late Fibrosis (S3-4) in Patients with a Chronic HBV Infection

Parameter	Coefficient	SE	p-value	OR (95% CI)
Univariate analysis				
miR-29a	–0.071	0.037	0.046	0.931 (0.866–0.902)
miR-29b	–0.178	0.76	0.019	0.837 (0.721–0.971)
miR-29c	–0.127	0.054	0.019	0.881 (0.792–0.980)
miR-143	–0.033	0.020	0.008	0.967 (0.631–0.805)
miR-223	–0.099	0.048	0.038	0.906 (0.825–0.995)
miR-21	–0.385	0.200	0.025	0.681 (0.460–0.808)
miR-374	–0.299	0.008	0.028	0.788 (0.773–0.804)
miR-16	0.202	0.154	0.190	1.224 (0.905–1.655)
PLT	–0.021	0.004	0.000	0.979 (0.972–0.987)
WBC	–0.427	0.117	0.000	0.653 (0.519–0.822)
ALB	–0.132	0.041	0.001	0.876 (0.809–0.949)
HBsAg, $\log_{10}$ IU/mL	–1.053	0.275	0.000	0.349 (0.204–0.598)
HBV DNA, $\log_{10}$ IU/mL	–0.302	0.077	0.000	0.739 (0.635–0.860)
Multivariate analysis				
miR-29a	–0.165	0.201.	0.011*	0.848 (0.584–0.913)
miR-29b	–0.244	0.292	0.404	0.784 (0.442–1.389)
miR-143	–0.064	0.066	0.019*	0.938 (0.608–0.948)
miR-223	–0.491	0.176	0.004*	0.612 (0.434–0.864)
miR-21	1.239	0.835	0.138	3.451 (0.670–17.741)
miR-374	–0.165	0.201	0.411	0.848 (0.572–1.257)
PLT	–0.349	0.124	0.005*	0.705 (0.553–0.900)
WBC	–1.246	1.383	0.368	0.288 (0.019–4.329)
ALB	–0.270	0.364	0.459	0.921 (0.803–1.057)
HBsAg, $\log_{10}$ IU/mL	–1.390	1.916	0.468	0.249 (0.006–10.650)
HBV DNA, $\log_{10}$ IU/mL	1.239	0.835	0.138	3.451 (0.671–17.741)

Univariate and multivariate logistic regression analyses were performed to assess factors associated with the progression of liver fibrosis from S0-S2 to S3-S4. A stepwise forward multivariate analysis including the studied miRNAs and clinical parameters, such as white blood cell (WBC) counts, platelet (PLT) counts, albumin (ALB) levels, hepatitis B surface antigen (HBsAg) levels, and hepatitis B virus (HBV) DNA load, was conducted with a probability of entry <0.05 and a probability of removal >0.1. A p-value <0.05 is considered statistically significant.
miRNA, microRNA; SE, standard error; OR, odds ratio; CI, confidence interval.
*Represent statistical significance in multivariate analysis.

S2 to S3-S4 was evaluated using ROC analysis. The AUC was 0.936 with sensitivity=87.47% and specificity=86.49% (Fig. 4). Similarly, the diagnostic performance of the established three-miRNA and PLT panel for discriminating fibrosis progression from S0-S1 to S2-S4 or from S0-S3 to S4 was also evaluated using ROC analysis and the AUC was 0.7482 with sensitivity=82.57% and specificity=79.59% and 0.9495 with sensitivity=93.57% and specificity=92.69%, respectively (data not shown).

As mentioned above, the AUC for distinguishing fibrosis progression from S0-S1 to S2-S4 was not as accurate as distinguishing fibrosis progression from S0-S2 to S3-S4. Although the AUC is 0.9495 for distinguishing fibrosis progression from S0-S3 to S4, the classification method is not common. Therefore, we emphasize its strengths in distinguishing early fibrosis (S0-S2) patients from late fibrosis (S3-S4). Comparison of the AUC of the three-miRNA and PLT panel with that of individual miRNAs revealed that the three-miRNA and PLT panel was superior to individual miRNAs in discriminating S0-S2 from S3-S4. We then compare the three-miRNA and PLT panel with aspartate aminotransferase to platelet patio index (APRI) and fibrosis 4 score (FIB-4), which were well established noninvasive markers. The results revealed that there were significant differences between the AUC values of the three-miRNA and PLT panel and APRI and FIB-4 (0.936 vs 0.740, 0.936 vs 0.854, respectively; p<0.001, p<0.001, respectively) (Fig. 4). These results indicated that the three-miRNA and PLT panel has a higher sensitivity and specificity for discriminating S0-S2 from S3-S4 than the biomarkers reflecting the extent of liver fibrosis in patients with chronic HBV infection.



Variable	AUC	SE*	p-value†	Cutoff value	95% CI
Panel	0.936	0.028	<0.001	1.8730	0.881-0.992
APRI	0.740	0.045	<0.001	0.6103	0.651-0.828
FIB-4	0.854	0.035	<0.001	0.7621	0.785-0.923

*Under the nonparametric assumption; †Null hypothesis: true area=0.5.

Fig. 4. The three-miRNA and platelet (PLT) panel is superior to the aspartate aminotransferase to platelet ratio index (APRI) and the fibrosis-4 score (FIB-4) for discriminating the progression of liver fibrosis from S0-S2 to S3-S4. An analysis of the receiver operating characteristic (ROC) curves for the three-miRNA and PLT panel (miR-29a, miR-143, miR-223, and PLT) was used to differentiate early fibrosis (S0-S2, n=69) from late fibrosis (S3-S4, n=54). Areas under the curves for APRI and FIB-4 were compared with the panel to differentiate early fibrosis (S0-S2) from late fibrosis (S3-S4) in patients with a chronic hepatitis B virus infection.
SE, standard error; CI, confidence interval.

5. Discriminant analysis for validating the predictive efficiency of prediction model

At last, the predictive power of prediction model included PLT, miR-29a, miR-143 and miR-223 for discerning fibrosis progression from S0-2 to S3-4 was also validated by discriminant analysis. It can be clearly seen from Table 3 that the overall predictive percentage was 89.4%. Also, it correctly classified 91.3% and 87.0% respectively in the group of S0-2 and S3-4 respectively.

DISCUSSION

Early diagnosis and sustained follow-up of the progression of hepatic fibrosis is essential in terms of preventing liver cirrhosis and end-stage hepatic disease. Liver biopsy is an invasive procedure associated with certain outcomes and has severe limitations; hence, there is a need for new diagnostic tools. The present study revealed that miR-143, miR-223, miR-21, miR-374, and miR-16 were significantly upregulated, whereas miR-29 family members were downregulated in patients with chronic HBV infection compared with HCs, which is consistent

Table 3. Classification Table of the Discriminant Analysis

Fibrosis stage	Group size (n=123)	Predicted liver fibrosis stage		
		S0-S2 (n=70)	S3-S4 (n=53)	Correct percentage, %
S0-S2	69	63	6	91.3
S3-S4	54	7	47	87.0
Overall percentage				89.4

The power of platelet, miR-29a, miR-143, and miR-223 in predicting the progression of fibrosis is shown. This procedure is designed to develop a set of discriminating functions that will help predict S0-S2 versus S3-S4 based on the values of other quantitative variables. One hundred twenty-three cases were used to develop a model to discriminate between S0-S2 versus S3-S4; four predictor variables were entered. Among the 123 observations used to fit the model, 89.4% were correctly classified.

with previous study that miR-21 were upregulated in cholestatic pediatric liver disease,[20] acute cardiac allograft transplantation model[21] and renal fibrosis,[22-24] miR-29a were upregulated in cholestatic pediatric liver disease20 and liver fibrosis,[6,25] and increased miR-16 expression induced by hepatitis C virus infection promotes liver fibrosis.[17] In addition, the studied miRNAs distinguished fibrosis progression from S0-S2 to S3-S4 in patients with chronic HBV infection except miR-16. Univariate and multivariate logistic regression analysis revealed the three-miRNA and PLT panel of miR-29a, miR-143, miR-223, and PLT with high diagnostic accuracy to distinguish S0-S2 from S3-S4 in patients. Moreover, the three-miRNA and PLT panel demonstrated a significantly higher diagnostic value than APRI and FIB-4 in late fibrosis patients. These results implicated the studied miRNAs as reliable early biomarkers and possible therapeutic tools or targets for fibrosis treatment.

In the current study, circulating and tissue miRNAs were not always consistent. It was reported that the liver secretes circulating exosomes during injury with increase in serum miR-122 and miR-192, and a corresponding decrease in hepatic expression.[26,27] Another possible explanation is that in response to hepatic injury, an intrahepatic loss of miRNAs is observed, whereby the circulating levels of miRNAs are massively increased.[28,29] The persistent injury is characterized by infiltration of the liver by immune cells, progressive loss of hepatocytes, proliferation of myofibroblasts, and accumulation of extracellular matrix.[30] Thus, with fibrosis progression, hepatic levels of miRNAs decrease and the release in the systemic circulation is also decreased, similar to that observed in our study for serum miR-143, miR-223, miR-21, and miR-374. Activation of HSCs, a key driver of fibrosis, is also associated with a specific miRNA deregulation regulating various fibrogenic signaling pathways,[31] whereas serum miR-29 family members were downregulated with fibrotic progression. A possible explanation is that the decreased secretion of miR-29 family members far exceeds the miR-29 family members passively released from hepatocytes or that passively released miR-29 family members do not possess a protective carrier and are readily degraded upon release. Therefore, liver miRNAs levels are needed. The discrepancies between different studies may also result from variability in technical procedures from sampling to detection method and data analysis, or the use of the different characteristics of the patient cohort. Liver cirrhosis etiology should also be considered.

Previous studies have evaluated circulating miRNAs as biomarkers of disease progression in liver fibrosis.[20,32-36] In hepatitis C patients, miR-182, miR199a-5p, miR-200a-5p, and miR-183 were found to be significantly upregulated in liver tissue with advanced fibrosis, stage F3 and F4, when compared with early fibrosis, stages F1 and F2.[32] Circulating miR-138 could serve as a noninvasive biomarker for the detection of early fibrosis, and miR-138 and miR-143 could be specific biomarkers for indicating the late stage of liver fibrosis in hepatitis C virus-related liver cirrhosis.[33,34] Similarly, circulating miR-21 and miR-29a levels appear suitable to serve as noninvasive diagnostic markers to differentiate biliary atresia from other cholestatic disease in infancy.[20] In patients with HBV infection, miRNA microarray hybridization revealed that 140 miRNAs were detected in the S1-S4 patient groups, and the numbers of miRNAs differentially expressed in the S1-S4 patient groups were 48, 97, 84, and 56, respectively, with 12 miRNAs differentially expressed at all stages.[35] Moreover, a miRNA panel (miR-1, miR-146a-5p, and miR-451a) in HBV-related liver cirrhosis patients could readily distinguish from the HCs with AUC values close to 1.0.[36] However, few miRNAs have been revealed as ideal candidate biomarkers in discriminating fibrosis progression from S0-S2 to S3-S4, which involves multiple steps and is the clinical pathway of most chronic HBV infection cases. Our study revealed that the selected miRNAs except miR-16 were significantly degraded in S3-S4 compared to S0-S2 and hepatic synthetic function (albumin and platelet) were reduced during liver disease progression from S0-S2 to S3-S4, raising the possibility that studied miRNAs could discriminate fibrosis progression from S0-S2 to S3-S4. Indeed, our study revealed miRNAs change significantly from early fibrosis to late fibrosis, and identified a four-miRNA set with high accuracy for discriminating fibrosis progression from S0-S2 to S3-S4, which could be a biomarker of fibrosis progression. Moreover, the miRNA panel has a higher sensitivity and specificity for discriminating S0-S2 from S3-S4 than the biomarkers reflecting the extent of liver fibrosis such as PLT, WBC and ALB in patients with chronic HBV infection. Our study has some limitations. First, patients recruited regionally from the same hospital might have specific genetic background, which may influence the expression of miRNAs; therefore, a multicenter study is needed to minimize the chance for bias. Second, the miRNA panel for distinguishing S0-S1 from S2-S4 was not as accurate as distinguishing S0-S2 from S3-S4, which is the most difficult point even when using a combination of biochemical parameters and ultrasonography.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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REFERENCES

1. Song Y, Wang F, Huang Q, Cao Y, Zhao Y, Yang C. MicroRNAs contribute to hepatocellular carcinoma. Mini Rev Med Chem 2015;15:459-466.
2. O'Reilly S. MicroRNAs in fibrosis: opportunities and challenges. Arthritis Res Ther 2016;18:11.
3. Ebrahimi A, Sadroddiny E. MicroRNAs in lung diseases: recent findings and their pathophysiological implications. Pulm Pharmacol Ther 2015;34:55-63.
4. Wang F, Chen C, Wang D. Circulating microRNAs in cardiovascular diseases: from biomarkers to therapeutic targets. Front Med 2014;8:404-418.
5. Hayes CN, Chayama K. MicroRNAs as biomarkers for liver disease and hepatocellular carcinoma. Int J Mol Sci 2016;17:280.
6. Huang C, Zheng JM, Cheng Q, et al. Serum microRNA-29 levels correlate with disease progression in patients with chronic hepatitis B virus infection. J Dig Dis 2014;15:614-621.
7. Ogawa T, Iizuka M, Sekiya Y, Yoshizato K, Ikeda K, Kawada N. Suppression of type I collagen production by microRNA-29b in cultured human stellate cells. Biochem Biophys Res Commun 2010;391:316-321.
8. Sekiya Y, Ogawa T, Yoshizato K, Ikeda K, Kawada N. Suppression of hepatic stellate cell activation by microRNA-29b. Biochem Biophys Res Commun 2011;412:74-79.
9. Wang Y, Liu J, Chen J, Feng T, Guo Q. MiR-29 mediates TGF-beta 1-induced extracellular matrix synthesis through activation of Wnt/beta-catenin pathway in human pulmonary fibroblasts. Technol Health Care 2015;23 Suppl 1:S119-S125.
10. Li J, Du S, Sheng X, et al. MicroRNA-29b inhibits endometrial fibrosis by regulating the Sp1-TGF-beta1/Smad-CTGF axis in a rat model. Reprod Sci. 2016;23:386-394.
11. Gabriely G, Wurdinger T, Kesari S, et al. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol 2008;28:5369-5380.
12. Zhao J, Tang N, Wu K, et al. MiR-21 simultaneously regulates ERK1 signaling in HSC activation and hepatocyte EMT in hepatic fibrosis. PLoS One 2014;9:e108005.
13. Wei J, Feng L, Li Z, Xu G, Fan X. MicroRNA-21 activates hepatic stellate cells via PTEN/Akt signaling. Biomed Pharmacother 2013;67:387-392.
14. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature 2008;454:56-61.
15. Zhang J, Jiao J, Cermelli S, et al. miR-21 inhibition reduces liver fibrosis and prevents tumor development by inducing apoptosis of CD24+ progenitor cells. Cancer Res 2015;75:1859-1867.
16. Naito Y, Sakamoto N, Oue N, et al. MicroRNA-143 regulates collagen type III expression in stromal fibroblasts of scirrhous type gastric cancer. Cancer Sci 2014;105:228-235.
17. Zhu B, Wei XX, Wang TB, Zhou YC, Liu AM, Zhang GW. Increased miR-16 expression induced by hepatitis C virus infection promotes liver fibrosis through downregulation of hepatocyte growth factor and Smad7. Arch Virol 2015;160:2043-2050.
18. Chim SS, Shing TK, Hung EC, et al. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 2008;54:482-490.
19. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513-1520.
20. Goldschmidt I, Thum T, Baumann U. Circulating miR-21 and miR-29a as markers of disease severity and etiology in cholestatic pediatric liver disease. J Clin Med 2016;5:pii.E28.
21. Gupta SK, Itagaki R, Zheng X, et al. miR-21 promotes fibrosis in an acute cardiac allograft transplantation model. Cardiovasc Res 2016;110:215-226.
22. Liu XJ, Hong Q, Wang Z, Yu YY, Zou X, Xu LH. MicroRNA21 promotes interstitial fibrosis via targeting DDAH1: a potential role in renal fibrosis. Mol Cell Biochem 2016;411:181-189.
23. McClelland AD, Herman-Edelstein M, Komers R, et al. miR-21 promotes renal fibrosis in diabetic nephropathy by targeting PTEN and SMAD7. Clin Sci (Lond) 2015;129:1237-1249.
24. Liu X, Hong Q, Wang Z, Yu Y, Zou X, Xu L. Transforming growth factor-beta-sphingosine kinase 1/S1P signaling upregulates microRNA-21 to promote fibrosis in renal tubular epithelial cells. Exp Biol Med (Maywood) 2016;241:265-272.
25. Hyun J, Jung Y. MicroRNAs in liver fibrosis: focusing on the interaction with hedgehog signaling. World J Gastroenterol 2016;22:6652-6662.
26. Povero D, Eguchi A, Li H, et al. Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. PLoS One 2014;9:e113651.
27. Shafaei S, Soleimani Amiri S, Hajiahmadi M, Sadeghi-Haddad-Zavareh M, Bayani M. Histological grading and staging of liver and its relation to viral loads in chronic anti-HBe positive hepatitis. Caspian J Intern Med 2013;4:681-685.
28. Wang K, Zhang S, Marzolf B, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci U S A 2009;106:4402-4407.
29. Cheung O, Puri P, Eicken C, et al. Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. Hepatology 2008;48:1810-1820.
30. Trebicka J, Anadol E, Elfimova N, et al. Hepatic and serum levels of miR-122 after chronic HCV-induced fibrosis. J Hepatol 2013;58:234-239.
31. Guo CJ, Pan Q, Cheng T, Jiang B, Chen GY, Li DG. Changes in microRNAs associated with hepatic stellate cell activation status identify signaling pathways. FEBS J 2009;276:5163-5176.
32. Van Keuren-Jensen KR, Malenica I, Courtright AL, et al. microRNA changes in liver tissue associated with fibrosis progression in patients with hepatitis C. Liver Int 2016;36:334-343.
33. El-Ahwany E, Nagy F, Zoheiry M, et al. Circulating miRNAs as predictor markers for activation of hepatic stellate cells and

progression of HCV-induced liver fibrosis. Electron Physician 2016;8:1804-1810.
34. Lee CH, Kim JH, Lee SW. The role of microRNA in pathogenesis and as markers of HCV chronic infection. Curr Drug Targets 2017;18:756-765.
35. Zhang Q, Xu M, Qu Y, et al Analysis of the differential expression of circulating microRNAs during the progression of hepatic fibrosis in patients with chronic hepatitis B virus infection. Mol Med Rep 2015;12:5647-5654.
36. Jin BX, Zhang YH, Jin WJ, et al. MicroRNA panels as disease biomarkers distinguishing hepatitis B virus infection caused hepatitis and liver cirrhosis. Sci Rep 2015;5:15026.