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ORIGINAL ARTICLE

Combination of MicroRNAs and Cytokines: a Method for Better Evaluation of Acute-on-Chronic Liver Failure

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SUMMARY

Background: **The aim was to establish expression profiles of microRNAs (miRNAs) in Peripheral Blood Mononuclear Cells (PBMC) and of plasma cytokines from the patients with hepatitis B virus related acute-on-chronic liver failure (HBV-ACLF) and high-risk of acute-on-chronic liver failure (ACLF) patients as well as healthy people and to examine the relationships between these profiles and clinical features.**
Methods: **Herein, we collected PBMCs and plasma from peripheral blood of HBV-ACLF and ACLF patients as well as healthy people. Microarray, real-time qPCR, and ELISA assays were used.**
Results**: In this study, we found 39 miRNAs including miR-146a, miR-150, and miR-29a downregulated and 5 miRNAs elevated in PBMCs from HBV-ACLF patients compared to healthy controls. However, elevated plasma levels of cytokines such as TNF-α, IFN-α, and TGF-β were found in PBMCs from HBV-ACLF patients compared with the controls, but no significant difference was found between the high-risk and control groups. MiR-146a, miR-150, miR-29a, PTA, and anti-HBc were positively correlated with the survival of ACLF patients, while TNF-α, IFN-α, INR, and TBiL were negatively correlated with the survival of these patients.**
Conclusions: **Combined examination of miRNAs in PBMCs and cytokines in plasma together is a better method for monitoring and evaluating HBV-ACLF patients.**
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KEY WORDS

HBV-ACLF, PBMC, microRNA, cytokine, clinical characteristics

LIST OF ABBREVIATIONS

$miRNA_S$ - $microRNA_S$
PBMC - peripheral blood mononuclear cells
ACLF - high-risk of acute-on-chronic liver failure
HBV-ACLF - hepatitis B virus related acute-on-chronic liver failure
PTA - prothrombin activity
PLT - platelet
INR - international normalized ratio
TBiL - total bilirubin
DBiL - direct bilirubin
Cr - creatinine

ALT - alanine aminotransferase
MELD - model for end-stage liver disease
Anti-HB_S - hepatitis B surface antibody
Anti-HB_C - hepatitis B core antibody
MS - multiple sclerosis
KC_S - Kupffer cells

INTRODUCTION

HBV-ACLF is an infectious disease that causes significant morbidity and mortality. In China, HBV-ACLF accounts for about 80% of all ACLF cases due to an immense population base of chronic HBV infection [1]. An evidence-based definition of ACLF is the presence of organ failure and high 28-day mortality (> 33%) [2]. ACLF displays key features of systemic inflammation, and its poor outcome is closely associated with exacerbated systemic inflammatory responses [3]. However, PBMCs and cytokines in plasma are key elements of the immune systems. For patients with ACLF, liver biopsy is impractical in clinical practice. To evaluate the relationships among miRNAs, cytokines and clinical characteristics may be a new way to diagnosis HBV-ACLF. MiRNAs are a set of small non-coding RNAs of 18 - 22 nucleotides that are important regulators of gene expression at the post-transcriptional level [4]. These miRNAs bind to the 3' untranslated region of specific messenger RNAs, degrading target mRNAs or repressing their translation, thereby affecting protein expression [4]. MiRNAs have been implicated in the regulation of many fundamental cellular and biological processes, such as cell differentiation, proliferation, apoptosis, and immune responses [5,6]. Cellular miRNAs also affect virus replication and pathogenesis. Studies showed that miRNAs participated in the process of liver injury. A pioneer study reported that serum miR-122 and miR-155 were positively associated with alcoholic and inflammatory liver injuries [7]. Although evaluation of miRNA expression has been used to diagnosis and evaluate human diseases, their values as biomarkers in PBMC for HBV-ACLF patients remain unexplored. Previous studies mainly focused on serum miRNAs that are often low in abundance and unstable for valuable analysis. The expression of serum miRNA has been negatively associated with ACLF patients, but which was consistent for microarray analysis. However, the miRNAs in PBMCs are surprisingly stable since they are important factors for immunity. Moreover, cytokines are proteins with pleiotropic functions and involved in proliferation, migration, adhesion, apoptosis, and immune modulation [8]. However, the relationships among miRNAs in PBMCs, downstream cytokines in plasma, and clinical characteristics have been rarely studied in HBV-ACLF patients.

In this study, we assessed miRNAs from PBMCs and cytokines from plasma extracted from HBV-ACLF patients and analyzed the clinical characteristics of patients.

MATERIALS AND METHODS

Patient characteristics

A total of 35 subjects were recruited from January, 2014 to December, 2015 from the Sixth People's Hospital of Dalian. The study is in line with relevant regulations of medical ethics. Baseline patient characteristics are displayed in Table 1. They were divided into three groups: HBV-ACLF (n = 12), high-risk (n = 11), and healthy controls (n = 12). HBV-ACLF was diagnosed according to the criteria set by the Asian Pacific Association for the Study of the Liver (history of CHB or cirrhosis; total bilirubin ≥ 85 mmol/L, prothrombin activity ≤ 40%). All patients with HBV-ACLF were followed up for at least six months. Survival period was based on six-month observation. The high-risk group was enrolled based on the following criteria: chronic history of HBsAg positive, elevations in normal ≤ ALT/AST levels ≤ 10 times, 40% ≤ prothrombin activity ≤ 80%. Healthy controls were individuals who were in healthy condition without detectable malignancy. Written informed consent was obtained from each participant before being enrolled into the study. The study protocol was approved by the Ethics Committee of Sixth People's Hospital of Dalian (Approval ID: 2014-003-02).

Collection of peripheral blood mononuclear cells (PBMCs)

Whole blood samples were collected in EDTA-treated tubes, and PBMCs were isolated using the lymphocyte separation medium (Tianjin Haoyang Biological Manufacture CO).

Total RNA isolation and quality analysis

Total RNA was extracted from PBMCs. Concentrations and purity of the RNA samples were assayed by electrophoresis and spectrophotometry with an optical density (OD) OD260/280 ≥ 1.6 and OD260/230 ≥ 1.0. RNA with a RNA integrity number (RIN) ≥ 5 was subjected to microarray analysis.

Microarray analyses

The microRNA microarray analysis was carried out by Super Biotek Company (Shanghai, China) using Phalanx Biotech Human MicroRNA OneArray HmiOA5.1. The initial dose of total RNA was 2.0 μg. Probes were designed based on Sanger database Ver 20.0, with a total of 2,539 probes, and each microRNA sequence has three repetitive probes to reduce error and improve the accuracy. GenePix™ 4 was used to obtain images. The data were transformed into visual figures using Rosetta Resolver® System (Rosetta Biosoftware). Fold change > 0.585 (or fold change 0.585) and p-values < 0.05 were considered statistically significant.

MiRNA quantification by qRT-PCR

U6 is a miRNA that exists as a housekeeper gene and was used as a reference for miRNA qRT-PCR. Primer sequences for miRNA (hsa-miR-29a-3P, hsa-miR-150-

5P, miR146a-5P, and U6) are shown in Table 2. A fixed volume of 2 μL of RNA solution from serum sample was used as input into the reverse transcription using miRNA-specific stem-loop primers, details in Table 3 (Super Biotek Co., Shanghai, China). The reaction was carried out in an ABI Vii7 Real Time PCR System (Applied Biosystems) at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Real-time PCR was carried out on an ABI Vii7 Real Time PCR System at 95°C for 6 minutes, followed by 50 cycles of 95°C for 10 seconds and 52°C for 10 seconds, 72°C for 30 seconds; data of miRNAs and U6 were calculated using the $2^{-\Delta\Delta Ct}$ method.

ELISA

Plasma levels of TNF-α, TGF-β, and IFN-α were quantified using enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instruction (TNF-α, TGF-β: Ray Biotech, IFN-α: eBioscience, USA). Results were calculated using the Flow Cytometry Pro software (eBioscience).

Statistical analysis

Measurement data between two groups were compared by Student's *t*-test or the Mann-Whitney U test. Nominal variables were expressed as number/percentage and compared using the chi-square test. Continuous variables were expressed as mean ± standard deviation (SD). The differences among groups were examined during Student's *t*-tests or the analysis of variance (ANOVA) tests. The possible relationship between variables was evaluated using Pearson's correlation coefficient. We examined different clinical characteristics and data was shown in Table 4. The absolute values of Pearson's correlation coefficient (|r|) > 0.8 indicate high correlation, |r| > 0.5 moderate, |r| > 0.3 low.

Statistical analyses were performed with the SPSS 17.0. p-values < 0.05 were regarded as statistically significant, shown as "*", while < 0.01 were regarded as highly statistically significant, shown as "**".

RESULTS

miRNA microarray analysis

Individuals subjected to microarray analysis were divided into two groups: HBV-ACLF and healthy controls (n = 5 in each group). MiRNA microarray was performed to detect miRNA expression in PBMCs of HBV-ACLF patients (as shown in Figure 1). The fold changes of microarray analysis are shown in Table 3. Cluster analysis indicated that 44 miRNAs had altered expression in PBMCs of the HBV-ACLF patients compared with the healthy controls without HBV according to log2 |fold changes| > 1.9 and p < 0.05. Five miRNAs (miR-4531, miR-4449, miR-143-3, miR-4792, and miR-1292-5P) were up-regulated while 39 miRNAs were down-regulated in which the expression levels of three miRNAs (miR-150-5P, miR-146a-5P, and miR-29a-3P) were down-regulated about 2-fold and thus were chosen for further analysis.

Decreased levels of miR-150-5P, miR-146a-5P, and miR-29a-3P in PBMCs of HBV-ACLF

The down regulation of three miRNAs (miR-150-5P, miR-146a-5P, and miR-29a-3P) in PBMCs from HBV-ACLF patients compared with the healthy controls was further confirmed using qRT-PCR; miR-150-5P, miR-146a-5P, and miR-29a-3P were down-regulated by 2.302-, 2.325-, and 1.919-fold, respectively, as shown in Figure 2.

Elevated levels of cytokines TNF-α, IFN-α, and TGF-β in HBV-ACLF patient plasma with down-regulated miR-150-5P, miR-146a-5P, and miR-29a-3P in their PBMCs

TNF-α, IFN-α, and TGF-β are predicted target proteins of miR-150-5P, miR-146a-5P, and miR-29a-3P according to the analysis using target-scan software. Previous research studied these miRNAs and cytokines [9,10], but none of these miRNAs and cytokines was studied simultaneously in PBMCs and plasma of HBV-ACLF patients.

ELISA analysis showed that TNF-α, IFN-α, and TGF-β were significantly up-regulated in HBV-ACLF patients in comparison with the healthy controls (p < 0.01) (as shown in Figure 3). IFN-α and TNF-α expressions were also elevated in HBV-ACLF patients compared to the high-risk patients (p < 0.01), but no significant difference was observed between the high-risk and the healthy control groups. The fibrosis inducing factor TGF-β expression was also enhanced in HBV-ACLF patients, similar to the high-risk patients compared to the healthy controls.

Correlations of the miRNAs in PBMCs, the cytokines in plasma, and the clinical characteristics

To correlate the levels of the miRNAs in PBMCs and the cytokines in plasma with the clinical characteristics of these patients, Pearson's correlation analysis was carried out.

Correlations of three inflammation related miRNAs (146a-5P, 150-5P, and 29a-3P) in PBMCs and three cytokines (TNF-α, IFN-α, and TGF-β) in plasma with clinical characteristics including PTA (prothrombin activity), PLT (platelet), INR (international normalized ratio), TBiL (total bilirubin), DBiL (direct bilirubin), Cr (creatinine), ALT (alanine aminotransferase), MELD (model for end-stage liver disease), survival period anti-HBs (hepatitis B surface antibody), and anti-HBc (hepatitis B core antibody) were performed. Pearson's correlation coefficient results and the p-values are shown in Table 4. Survival period exhibited a close correlation with inflammatory biomarkers in ACLF patients. In this study, IFN-α and TNF-α were negatively and moderately correlated with survival period (r = -0.606**, -0.784**); miRNAs 146a-5P, 150-5P, and 29a-3P were moderately correlated with survival period (r = 0.541*,

Table 1. Clinical Characteristics of individuals.

Clinical parameter	Healthy	High-risk	ACLF
Age (years)	34.30 ± 8.77	45.82 ± 10.33	48.07 ± 9.34
Gender (M/F)	8/4	10/1	9/3
ALT (IU/L)	29.67 ± 31.02	125.71 ± 122.75	165.53 ± 257.08
AST (IU/L)	21.22 ± 8.60	168.373 ± 171.25	249.59 ± 349.12
R-GT (IU/L)	32.44 ± 39.96	64.76 ± 39.07	41.98 ± 23.32
PTA (%)	126 ± 10.31	56.43 ± 12.16	23.46 ± 9.97
TBiL (μmol/L)	11.86 ± 3.49	99.58 ± 53.51	364.60 ± 183.76
Cr (μmol/L)	63.11 ± 11.84	61.33 ± 13.44	140.61 ± 108.20
WBC (x 10^9/L)	5.99 ± 1.74	7.49 ± 4.78	9.06 ± 5.29
Survival period (days)	365 ± 0	365 ± 0	75 ± 100.17
HBsAg (IU/mL)	0.42 ± 0.07	213.38 ± 177.24	171.16 ± 170.55
anti-HBc (S/CO)	1.27 ± 0.84	0.70 ± 1.18	0.05 ± 0.06
MELD	6.46 ± 0.11	16.71 ± 2.83	34.33 ± 11.11

Notes: The data in the table represent the mean ± standard deviation.

Table 2. Primers of miRNAs used for qRT-PCR.

Primer	Sequence (5' to 3')
miR-29a-3p-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTAACCG
miR-29a-3p-F	TAGCACCATCTGAAATCGG
miR-150-5p-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCACTGG
miR-150-5p-F	TCTCCCAACCCTTGTACC
miR146a-5P-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAACCCA
miR146a-5P-F	TGAGAACTGAATTCCATGGG
U6 RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAAAATATG
U6-F	ATTAGCATGGCCCCTGCG
mMiRNA primer-R	TGCGTGTCGTGGAGTCG

0.535*, 0.581*). PTA and anti-HBc were moderately correlated with survival period (r = 0.683** and 0.409*). INR, TBiL, DBiL, and Cr were all negatively and moderately correlated with survival period (r = -0.712**, -0.732**, -0.653**, and -0.419*).

Some correlations were also found between the miRNAs and cytokines. TGF-β was negatively correlated with 146a-5P, 150-5P, and 29a-3P (r = -0.639*, -0.570* and -0.506). IFN-α was highly correlated with TNF-α (r = 0.742*). MiRNA 146a-5P was highly correlated with miRNAs 150-5P and 29a-3P (r = 0.855**, 0.881**), and miRNA 150-5P was highly correlated with 29a-3P (r = 0.924**).

TBiL is considered as a standard to define ACLF. TBiL was positively correlated with IFN-α, TNF-α, INR, and Cr (r = 0.520**, 0.735**, 0.732**, and 0.673**, respectively). TBiL was negatively correlated with miRNAs 146a-5P, 150-5P, 29a-3P, PTA, PLT, and anti-HBc (r = -0.620*, -0.617*, -0.589*, -0.716**, -0.483**, and -0.409*, respectively).

Coagulation characteristics (PTA, PLT, and INR) are also important clinical features of ACLF patients. PTA was correlated with miRNAs 146a-5P, 150-5P, 29a-3P, PLT, and anti-HBc (r = 0.872**, 0.828**, 0.772**, 0.803**, and 0.527**). PTA was negatively correlated with TNF-α, INR, TBiL, and DBiL (r = -0.509**, -0.713**, 0.716**, and 0.691**). PLT was positively correlated with 146a-5P and 150-5P (r = 0.689*, 0.676*). PLT was negatively correlated with TBiL and DBiL (r = -0.483**, -0.477**).

Table 3. Properties of miRNAs differentially expressed in the patients with HBV-ACLF compared with healthy controls.

miRNA	log2 (Ratio)	p-value	miRNA	log2 (Ratio)	p-value
miR-29a-3p	1.919	0.001	miR-4259	0.927	0.033
miR-150-5p	2.302	0.026	miR-3907	1.171	0.003
miR-146a-5p	2.325	0.038	miR-3135b	1.465	0.000
let-7f-5p	1.377	0.021	miR-4638-5p	0.923	0.016
let-7g-5p	1.698	0.018	miR-4690-5p	0.840	0.015
miR-557	0.959	0.002	miR-4776-5p	0.915	0.009
miR-26b-5p	0.807	0.017	miR-3607-5p	1.725	0.005
miR-342-3p	0.961	0.000	miR-4633-5p	0.851	0.004
miR-26a-5p	1.041	0.007	miR-4791	0.836	0.002
miR-146b-5p	2.071	0.037	miR-3607-3p	1.012	0.034
miR-769-5p	0.827	0.011	miR-3653	1.036	0.002
miR-142-5p	1.002	0.022	miR-664b-5p	0.997	0.024
miR-664a-5p	1.043	0.012	miR-5195-5p	1.001	0.014
miR-29b-3p	1.584	0.000	miR-1185-2-3p	1.023	0.008
miR-30d-5p	0.983	0.026	miR-1185-1-3p	0.962	0.000
let-7c	0.826	0.002	miR-6511b-5p	0.830	0.038
miR-885-3p	0.955	0.005	miR-6131	0.923	0.001
miR-142-3p	1.525	0.001	miR-1292-5p	0.991	0.008
miR-1248	0.999	0.007	miR-143-3p	0.883	0.017
miR-1915-3p	0.854	0.038	miR-4449	0.844	0.003
miR-1193	0.899	0.003	miR-4792	0.930	0.015
miR-126-3p	0.936	0.035	miR-4531	0.812	0.000

DISCUSSION

HBV-ACLF is an increasingly recognized disease characterized by an acute deterioration of liver function in patients with cirrhosis. ACLF displays key features of systemic inflammation that contributes to its poor outcome [11]. Immune cells secrete cytokines leading to hepatocyte apoptosis and eventually liver failure. The leukocytes residing in the liver and PBMCs in blood are very important mediators, and miRNAs in PBMCs are essential in the regulation of innate and adaptive immunity. This study was designed to evaluate the relationships among miRNAs, cytokines, and clinical characteristics of the HBV-ACLF and high-risk patients.

The relationship between miRNAs and the prognosis of acute liver failure was reported. Qing et al. speculated that up-regulation of miRNA-130 presents a good prognosis factor for HBV-ACLF patients [12]. Up-regulation of miR-122 could be detected before obvious histopathologic changes in the liver [13]. These results suggested that miRNAs could be used as biomarkers for diagnosis and monitoring this disease at early stages. In this study, we employed microarray analysis of PBMCs to seek the miRNAs that are possibly involved in the pathogenesis of HBV-ACLF. Then, three down-regulated miRNAs including miR-29a-3P, miR-146a-5P, and miR-150-5P were validated with the qRT-PCR method. We found these miRNAs were down-regulated in the HBV-ACLF group compared to the healthy control group while no difference between the high-risk group and the ACLF group was observed. These results suggested that the miRNAs miR-29a-3P, miR-146a-5P, and miR-150-5P might be developed into novel prognostic biomarkers for HBV-ACLF.

Cytokines are the important features of the innate immune response. The cytokine storm is the principal mechanisms underlying the systemic inflammatory response in ACLF [2]. TNF is a cytokine that is closely related with ACLF as reported in many studies. It has been reported that miR-146a-5P transcription is regulated by NF-κB, and its target genes such as TNF receptor-associated factor-6 (TRAF-6) are involved in the immune response [2]. Meanwhile, miR-146a-5P was identified as a spot miRNA during research of autoimmune diseases caused by immune disorder [2,14,15]. Researchers also demonstrated miR-29 suppressed IFN-γ production by directly targeting IFN-γ mRNA [10]. Up regulation of IFN-beta-responsive genes is accompanied by a down-regulation of the miR-29 family during the treatment of multiple sclerosis (MS) [16]. In this study,

Table 4. Pearson's correlation coefficient results and the p-values.

	Inflammation protein			miRNA			Survival period	Clinical characteristics							
	TGFβ	IFNα	TNFα	146a-5P	150-5P	29a-3P		PLT	INR	TBiL	DBiL	Cr	Anti-HBs	Anti-HBc	ALT
TGFβ	1	0.396	0.188	-0.639 *	-0.570 *	-0.506	-0.324	-0.456	0.093	0.296	0.279	-0.072	-0.242	0.149	0.567 *
		0.104	0.456	0.010	0.027	0.054	0.205	0.088	0.753	0.284	0.314	0.800	0.385	0.595	0.028
IFNα	0.396	1	0.742 **	-0.266	-0.277	-0.268	-0.606 **	-0.086	0.424 *	0.520 **	0.458 *	0.544 **	-0.117	-0.229	-0.018
	0.104		0.000	0.338	0.317	0.335	0.000	0.683	0.039	0.008	0.021	0.005	0.585	0.281	0.930
TNFα	0.188	0.742 **	1	-0.322	-0.350	-0.319	-0.784 **	-0.205	0.626 **	0.735 **	0.658 **	0.619 **	-0.146	-0.399 *	-0.069
	0.456	0.000		0.242	0.201	0.246	0.000	0.305	0.001	0.000	0.000	0.001	0.476	0.043	0.732
146a-5P	-0.639 *	-0.266	-0.322	1	0.855 **	0.881 **	0.541 *	0.689 **	-0.685 *	-0.620 *	-0.584 *	-0.189	0.686 **	0.484	-0.193
	0.010	0.338	0.242		0.000	0.000	0.046	0.009	0.014	0.024	0.036	0.535	0.010	0.094	0.528
150-5P	-0.570*	-0.277	-0.350	0.855 **	1	0.924 **	0.535 *	0.676 *	-0.703 *	-0.617 *	-0.577 *	-0.433	0.196	0.594 *	-0.227
	0.027	0.317	0.201	0.000		0.000	0.049	0.011	0.011	0.025	0.039	0.139	0.521	0.032	0.455
29a-3P	-0.506	-0.268	-0.319	0.881 **	0.924 **	1	0.581 *	0.543	-0.694 *	-0.589 *	-0.496	-0.408	0.336	0.609 *	-0.209
	0.054	0.335	0.246	0.000	0.000		0.029	0.055	0.012	0.034	0.085	0.167	0.261	0.027	0.494
Survival period	-0.324	-0.606 **	0.784 **	0.541 *	0.535 *	0.581 *	1	0.335	-0.712 **	-0.732 **	-0.653 **	-0.419 *	0.162	0.409 *	0.087
	0.205	0.000	0.000	0.046	0.049	0.029		0.061	0.000	0.000	0.000	0.017	0.392	0.025	0.635
PTA (%)	-0.420	-0.292	-0.509 **	0.872 **	0.828 **	0.772 **	0.683 **	0.803 **	-0.713 **	-0.716 **	-0.691 **	-0.431 *	0.409 *	0.527 **	0.014
	0.119	0.157	0.007	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.011	0.020	0.002	0.939

Table 4. Pearson's correlation coefficient results and the p-values (continued).

	Inflammation protein			miRNA			Survival period	Clinical characteristics							
	TGFβ	IFNα	TNFα	146a-5P	150-5P	29a-3P		PLT	INR	TBiL	DBiL	Cr	Anti-HBs	Anti-HBc	ALT
PLT	-0.456	-0.086	-0.205	0.689**	0.676*	0.543	0.335	1	-0.341	-0.483**	-0.477**	-0.282	0.371*	0.317	0.001
	0.088	0.683	0.305	0.009	0.011	0.055	0.061		0.052	0.004	0.004	0.106	0.037	0.077	0.997
INR	0.093	0.424*	0.626**	-0.685*	-0.703*	-0.694*	-0.712**	-0.341	1	0.732**	0.658**	0.773**	-0.223	-0.347	-0.049
	0.753	0.039	0.001	0.014	0.011	0.012	0.000	0.052		0.000	0.000	0.000	0.229	0.056	0.788
TBiL	0.296	0.520**	0.735**	-0.620*	-0.617*	-0.589*	-0.732**	-0.483**	0.732**	1	0.985**	0.673**	-0.263	-0.409*	-0.069
	0.284	0.008	0.000	0.024	0.025	0.034	0.000	0.004	0.000		0.000	0.000	0.146	0.020	0.697
DBiL	0.279	0.458*	0.658**	-0.584*	-0.577*	-0.496	-0.653**	-0.477**	0.658**	0.985**	1	0.617**	-0.276	-0.379*	-0.006
	0.314	0.021	0.000	0.036	0.039	0.085	0.000	0.004	0.000	0.000		0.000	0.127	0.033	0.975
Cr	-0.072	0.544**	0.619**	-0.189	-0.433	-0.408	-0.419*	-0.282	0.773**	0.673**	0.617**	1	-0.102	-0.254	-0.060
	0.800	0.005	0.001	0.535	0.139	0.167	0.017	0.106	0.000	0.000	0.000		0.579	0.161	0.735
Anti-HBs	-0.242	-0.117	-0.146	0.686**	0.196	0.336	0.162	0.371*	-0.223	-0.263	-0.276	-0.102	1	0.079	-0.104
	0.385	0.585	0.476	0.010	0.521	0.261	0.392	0.037	0.229	0.146	0.127	0.579		0.669	0.570
Anti-HBc	0.149	-0.229	-0.399*	0.484	0.594*	0.609*	0.409*	0.317	-0.347	-0.409*	-0.379*	-0.254	0.079	1	0.323
	0.595	0.281	0.043	0.094	0.032	0.027	0.025	0.077	0.056	0.020	0.033	0.161	0.669		0.071
ALT	0.567*	-0.018	-0.069	-0.193	-0.227	-0.209	0.087	0.001	-0.049	-0.069	-0.006	-0.060	-0.104	0.323	1
	0.028	0.930	0.732	0.528	0.455	0.494	0.635	0.997	0.788	0.697	0.975	0.735	0.570	0.071	

Notes: * - Correlation is significant at the 0.05 level (2-tailed). ** - Correlation is highly significant at the 0.01 level (2-tailed).

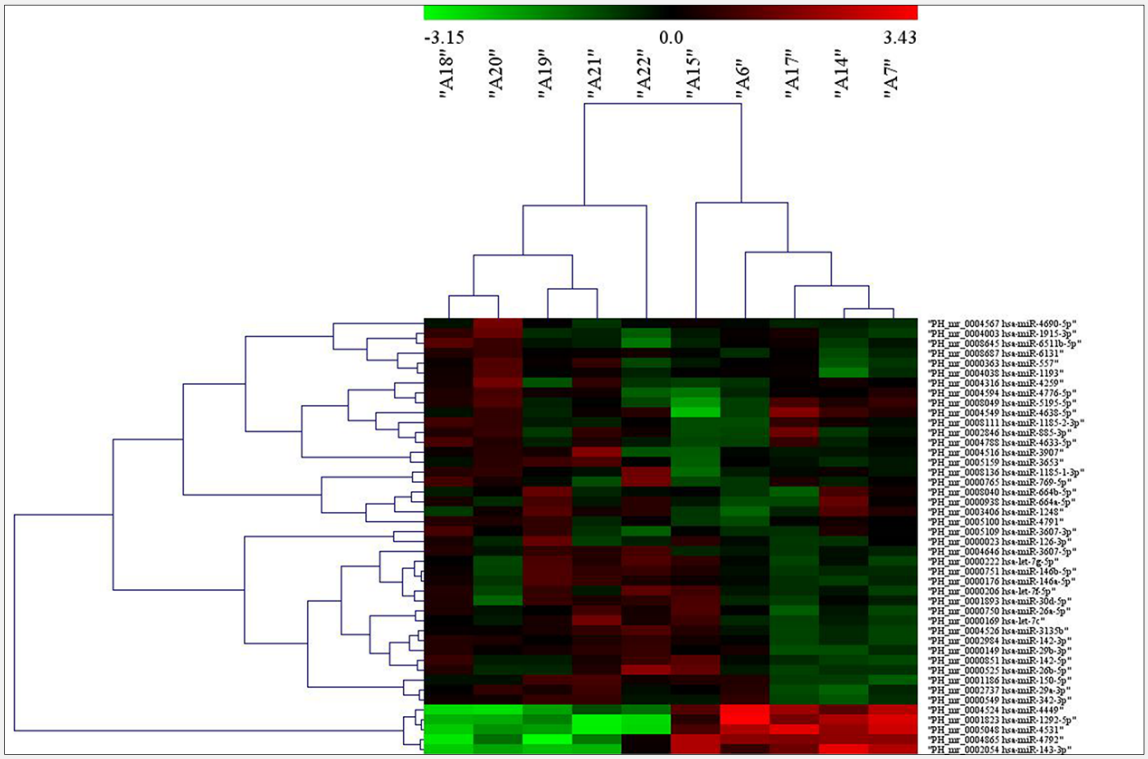

Figure 1. The clustering results for miRNA microarray of the samples.

Red and green color scale indicated aberrantly expressed miRNAs in the HBV-ACLF and control groups, A18 - A7 were different patient numbers, where A18, A20, A19, A21, and A22 were control groups, the others were the HBV-ACLF group.

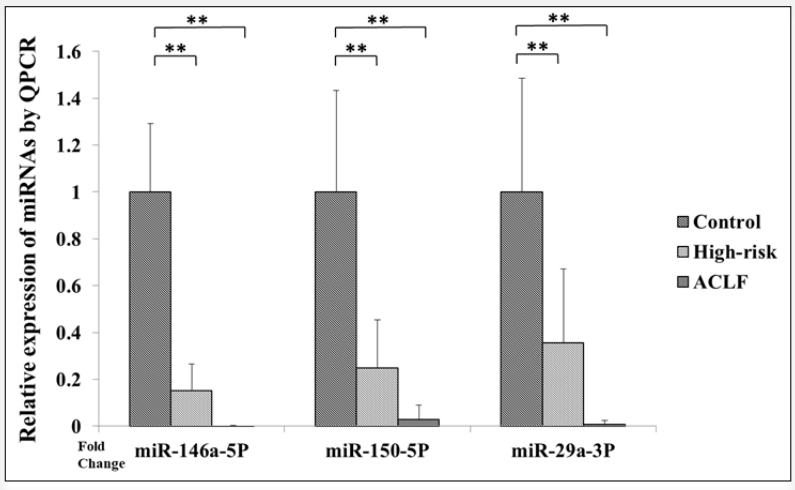

Figure 2. Qualitative analysis results of qRT-PCR of miRNA in PBMCs for patients with HBV-ACLF, high-risk, and healthy controls.

**** - p < 0.01.**

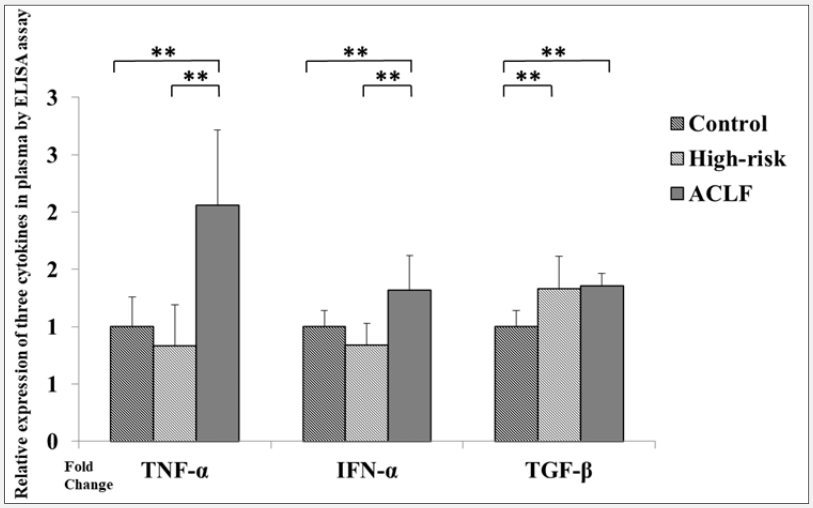

Figure 3. The relative expression of TNF-α, IFN-α, and TGF-β in HBV-ACLF, high-risk, and healthy groups by ELISA assay.

Unit was ng/mL. ** - $p < 0.01$.

we found that miR-146a-5P, miR-150-5P, and miR-29a-3P in PBMCs were down-regulated, while TNF-α and IFN-α in plasma were elevated in HBV-ACLF patients compared to the healthy control and high-risk group, suggesting that these three miRNAs may be involved in inflammatory responses of HBV-ACLF patients.

Liver fibrosis is the result of an exacerbated wound-healing response associated with chronic liver injury. Advanced liver fibrosis results in cirrhosis and liver failure. Cumulative evidence suggested that miRNAs might be involved in the pathogenesis of liver fibrosis [17]. Studies indicated that miR-150-5P expression was significantly reduced during liver fibrosis [18]. MiR-150-5P also takes part in pathogen infections and autoimmune diseases [19]. HBV infection induced TGF-β production induces fibrosis via T regulatory lymphocytes and hepatic stellate cells [20]. Elevation of miR-29-3P expression leads to down-regulation of collagen mRNA expression [10]. It has been reported that miR-29-3P is regulated by TGF-β and its expression prevents fibrosis by avoiding extracellular matrix deposition in renal parenchyma [21]. Thus miR-150-5P and miR-29-3P regulate immune function and fiber deposits as well. In this study, these two microRNAs were all down-regulated in HBV-ACLF patients compared with the healthy control and high-risk group. In addition, TGF-β expression was elevated in the HBV-ACLF group compared to the control group, but no difference was found between the HBV-ACLF and high-risk groups. It was speculated that down-regulated miRNAs led to fiber accumulation in the liver, causing liver blood circulation disorder, which led to insufficient blood supply for liver cells.

In this study, our Pearson's correlation analysis demonstrated some interesting correlations between these biomolecules and patients' clinical characteristics. Our results indicated that the expression of the three miRNAs we studied were all moderately correlated with PLT and survival period, and miR-150-5P and miR-146a-5P expression correlated positively with PLT. We also found that TBiL was inversely correlated with IFN-α and TNF-α expression as well, and it was negatively correlated with miRNA 146a-5P, 150-5P, 29a-3P, PTA, PLT, and anti-HBc, suggesting that platelets are involved in immune regulation. A previous study showed that PLT associated with survival in a mouse model of ALF. PLTs interact with Kupffer cells (KCs) in this model and exert their beneficial effect through reduction of oxidative stress, which eventually protects hepatocytes from apoptosis [22]. Correlations among these miRNAs suggest that the expression of these miRNAs may be regulated by the same network. Thus, evaluation of these biomarkers simultaneously may be an important approach for more accurate prognosis of liver failure. Although significant progress has been made in the field of HBV-ACLF during the past several years, high mortality still exists. Our results that inflammation-related miR-146a-5P, miR-150-5P, and miR-29a-3P were closely associated with HBV-ACLF, suggesting that a combination of miRNAs (miR-29a-3P, miR-146a-5P, and miR-150-5P) in PBMCs and cytokines (TNF-α, IFN-α, and TGF-β) in plasma is a good approach to diagnose HBV-ACLF and evaluate patients' status more

accurately. PBMC miRNAs and plasma cytokines were studied in the HBV-ACLF and high-risk group for the first time. More extensive future studies with more patients are required to verify our results. However, our study provides a novel approach for more accurate diagnosis of HBV-ACLF by evaluating the immune status of HBV-ACLF patients.

CONCLUSION

In this study, we found that inflammation-related miRNAs, miR-29a-3P, miR-146a-5P, and miR-150-5P were negatively associated with HBV-ACLF, while inflammatory factors such as TNF-α and IFN-α were positively associated with HBV-ACLF. Thus, evaluation of blood inflammation-related miRNAs together with cytokines could provide more information about inflammation status of HBV-ACLF patients, which may be developed into a new liver biopsy method and may be an invaluable approach to guide the effective management of HBV-ACLF.

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Declaration of Interest:
The authors declare no conflict of interest.

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