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ORIGINAL ARTICLE

MicroRNA Profile in Peripheral Blood Mononuclear Cells from Hepatitis B Virus Infected Patients

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ABSTRACT

Introduction and aim. The pathogenesis of hepatitis B virus (HBV)-related liver diseases remains not fully understood. Here, we aim to explore the potential roles of dysregulated miRNAs in chronic hepatitis B (CHB) and HBV-related acute-on-chronic liver failure (ACLF). **Material and methods.** MiRNA microarray was conducted in peripheral blood mononuclear cells (PBMCs) obtained from healthy donors or patients with CHB or ACLF. Altered expression of miRNAs was further confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Finally, the differentially expressed miRNAs and their target genes were subjected to bioinformatics analysis. **Results.** The miRNA microarray identified 45 up-regulated and 62 down-regulated miRNAs with a fold change ≥ 1.5. Expression of eight miRNAs was validated using qRT-PCR analysis, which was consistent with miRNA microarray analysis. Bioinformatics analysis indicated that multiple biological processes and signaling pathways were affected by these miRNAs and a miRNA-gene regulatory network was generated with Cytoscape. **Conclusion.** The current study provided a global view of miRNA expression in PBMCs from CHB and ACLF patients. Functional analysis showed that multiple biological processes and signaling pathways were modulated by these miRNAs. These data provide intriguing insights into the molecular pathogenesis of HBV-related liver diseases, which deserve further investigation.

Key words. Non-coding RNA. Chronic hepatitis B. Bioinformatics.

INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem. Two billion people worldwide have been infected with HBV, and among them more than 240 million are chronic HBV carriers.[1] This results in about 650,000 deaths of HBV related liver failure, cirrhosis, and hepatocellular carcinoma annually,[2] which bring huge healthcare cost and socioeconomic burdens.

MicroRNAs (miRNAs), as a class of small non-coding molecules approximately 22 nt in length, play critical roles in the post-transcriptional regulation of gene expression either by repressing translation initiation and protein synthesis, or by inducing degradation of messenger RNA (mRNA).[3,4] Evidence indicates that miRNAs play a fundamental role in the control of many biological processes, such as cellular development, differentiation, proliferation, apoptosis, and metabolism,[5] and many studies have reported a potential link between miRNAs and human diseases.[6-8] In HBV-related liver diseases, researches concerning miRNAs mainly focused on hepatocellular carcinoma.[6,9-11] In addition, Ji, *et al.*[12] investigated the differentially expressed miRNAs in serum during HBV infection and underscored the potential importance of miR-122 in the infection process. More importantly, it is reported that in human lupus, overexpression of miR-146a can reduce the induction of type I interferon (IFN) in peripheral blood mononuclear cells (PBMCs),[13] which are the major extrahepatic milieu of infection and viral replication, meantime, is a critical component in the immune system to fight against infection and adapt to intruders. Therefore, to elucidate the pathogenesis of hepatitis B, a better understand of the miRNA expression profile and their biological function in PBMCs in

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patients with chronic hepatitis B virus infection is of great importance.

In this study, we described the differentially expressed miRNAs in PBMCs from chronic hepatits B (CHB) and HBV-related acute-on-chronic liver failure (ACLF) patients, and identified their potential targets. A subset of differentially expressed miRNAs was validated by quantitative real-time polymerase chain reaction (qRT-PCR). We also analyzed the biological function of these miRNAs through bioinformatic analysis. A regulatory network of miRNA-mRNA was constructed to provide new insights into the pathogenesis of hepatitis B.

MATERIALS AND METHODS

Participants

Blood samples were collected from healthy donors (n = 32) or patients with CHB (n = 28) or ACLF (n = 6) that met the eligibility criteria.[14] In particular, the ACLF patients had a history of CHB, with serum total bilirubin more than five times the upper limit of normal (≥ 85 μmol/L), prothrombin activity < 40%, recent occurrence of hepatic encephalopathy (≥ grade II), plus ascites or hepato-renal syndrome. They were also negative for other viruses, e.g., hepatitis C virus, hepatitis D virus and human immunodeficiency virus. Patients had not received any anti-viral treatment or immunotherapy for 6 months prior to blood collection. Healthy donors (n = 20) (age-, sex- and race-matched) had no evidence of prior exposure to HBV (negative for hepatitis B surface antigen). The investigational protocol was approved by the Human Ethics Committee of Shanghai Fudan University, and informed consents were obtained from all study participants.

Generation of Peripheral blood mononuclear cells (PBMCs)

Blood samples were collected in sterile Vacutainer tubes (BD, Franklin Lakes, NJ) with liquid K3 EDTA. PBMCs from the participants were isolated from 10 mL EDTA-anticoagulated blood samples by density gradient separation using Lympholyte-H (Cedar-lane Laboratories, Ltd., Ontario, Canada).

miRNA extraction and reverse transcription

Total RNA, including miRNAs, was extracted from PBMCs and purified using the miRNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA concentration was quantified using a NanoDrop spectrophotometer (Thermo, Fisher Sci-entific). The integrity and quality of RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies). An aliquot of 0.25 μg of total RNA was converted to cDNA using the miScript RT kit (Qiagen).

Microarray analysis

A total of nine RNA samples from three healthy donors, three CHB patients, and three ACLF patients were further detected by microarray at Guangzhou Ribo, Co., Ltd., using RiboArray miDETECT Human Array 1 x 12K, which contains 2042 human miRNA sequences from the miRBase19.0 database. Labeling and hybridization were performed according to the manufacturer's protocols. Signals on the slides were scanned using an Agilent G2565BA microarray scanner (Agilent Technologies, Palo Alto, CA). For each sample, triplicates were analyzed at each group for a given detectable miRNA signal. The ratio of the two sets of detected signals (log 2 transformed, balanced) and the P-values of the t-test and ANOVA were calculated. The differentially detected signals with $P < 0.05$ were analyzed using gene hierarchical clustering of the log 2 value of each different time group and then were displayed in a heatmap. Clustering was performed using Cluster 3.0 created by Michiel de Hoon, Seiya Imoto, and Satoru Miyano, University of Tokyo, Human Genome Center (Euclidean distance, links using the average) and viewed in heatmap using Java TreeView 1.0.13 software.

qRT-PCR analysis

qRT-PCR was performed using a miScript SYBR Green PCR kit (Qiagen) according to the manufacturer's directions on an Mx3000p RT-PCR platform (Stratagene). A universal reverse primer was provided by the manufacturer, and each forward primer was specific to the mature miRNA listed in the Sanger miRBase. The expression of miRNAs was normalized using the U6 small RNA as the endogenous control. The relative amounts of PCR product were determined using the comparative Ct method ($2^{-\Delta\Delta CT}$).

Data processing

Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) annotation was then performed on the target genes of selected miRNAs by The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/list.jsp). Network of dysregulated miRNAs and their target genes was presented by Cytoscape (Version 3.4.0). The integrative KEGG pathway and GO term analysis was performed with the Cytoscape plugin ClueGO.[15]

Statistical analysis

Data are expressed as mean ± SD. Differences were compared by one-way ANOVA. A value of $P < 0.05$ was considered statistically significant. All experiments were performed at least three times.

RESULTS

miRNAs differentialy expressed in PBMCs from patients with CHB/ ACLF

To identify the miRNA spectra, we used a high-throughput miRNA array to compare the miRNA profiles of the HC, CHB and ACLF groups. Among the aberrantly expressed microRNAs, 107 microRNAs (45 up-regulated and 62 down-regulated) were identified with a fold change ≥ 1.5. Hierarchical clustering was performed on these miRNAs, and a heatmap was presented to visualize the results (Figure 1). To further validate these results, eight miRNAs (miR-548an, miR-548i, miR-4462b, miR-4735-3p, miR-550a-3p, miR-944, miR-1471 and miR-4281) with the most significant fold change were subjected to qRT-PCR analysis, which showed consistent results with the miRNA array analysis (Figure 2). The demographic and clinical features of patients and healthy individuals are detailed in table 1.

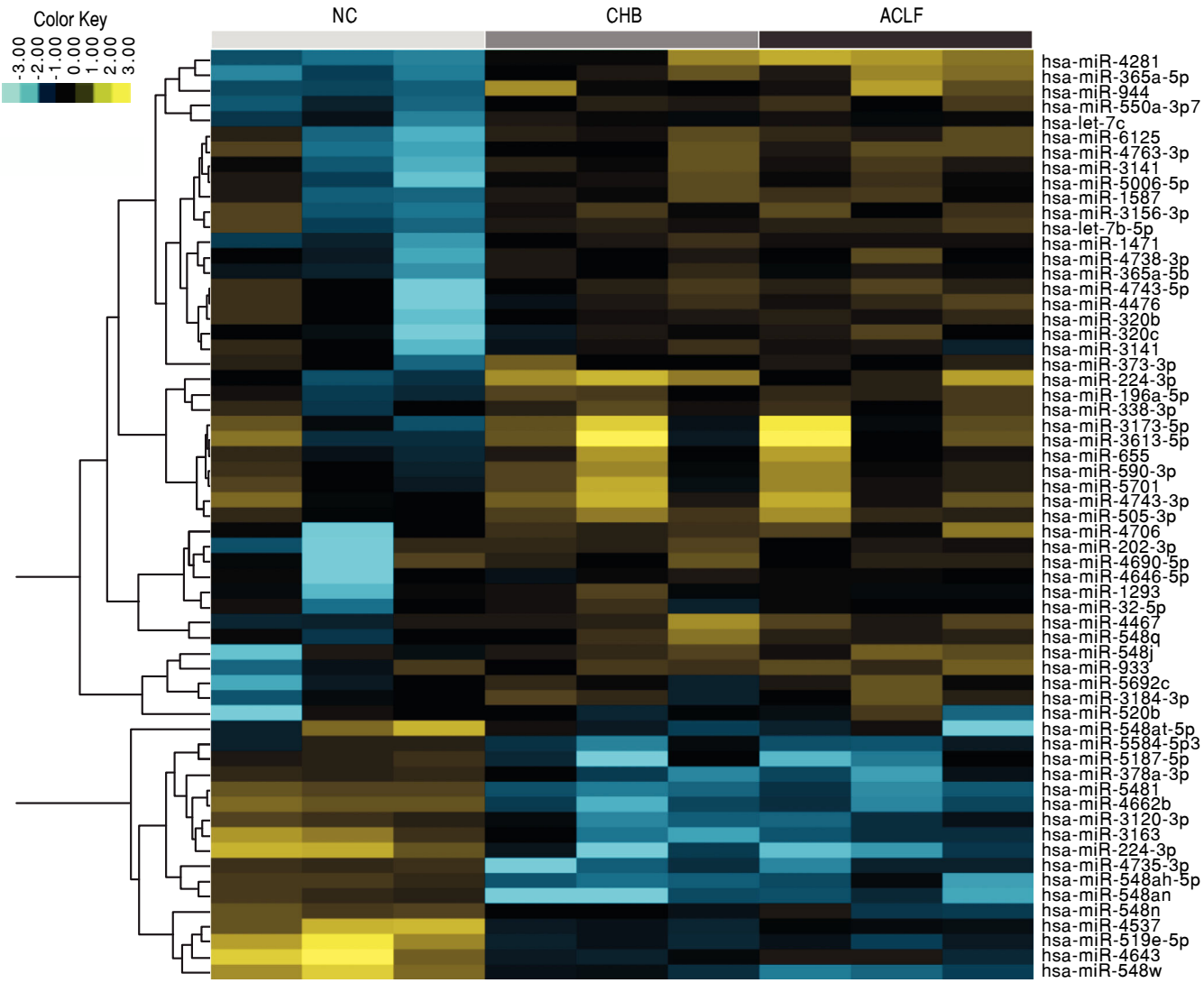

Figure 1. *Hierarchical cluster analysis of the 107 selected miRNAs from health controls, patients with chronic hepatitis B and acute-on-chronic liver failure (ACLF). The heatmap represents color-coded relative expression values in which yellow values indicate over-expression and blue values indicate under-expression. NC: healthy donor. CHB: chronic hepatitis B. ACLF: HBV-related acute-on-chronic liver failure.*

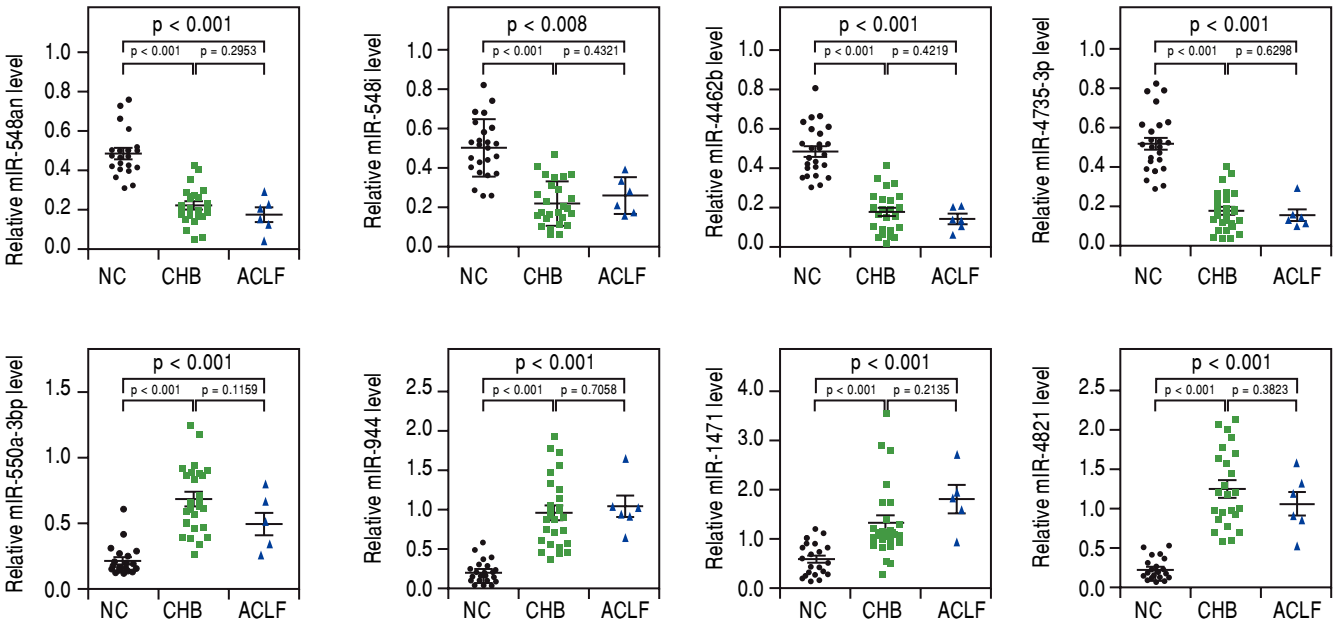

Figure 2. *qRT-PCR analysis of the relative expression levels of eight microRNAs as indicated. p < 0.05 was considered statistically significant. NC: healthy donor. CHB: chronic hepatitis B. ACLF: HBV-related acute-on-chronic liver failure.*

Table1. Clinical characteristics of the study subjects.

Clinical parameters	HC (n = 32)	CHB (n = 28)	ACLF (n = 6)
Gender(m/f)	17/15	21/7	3/3
Age (yr)	33.9 ± 11.4	36.1 ± 9.5	48.3 ± 12.4
ALT(IU/L)	22.8 ± 6.0	41.5 ± 5.5	41.7 ± 11.3
AST(IU/L)	20.5 ± 4.6	50.5 ± 21.0	93.7 ± 16.0
ALB(IU/L)	44.6 ± 3.2	42.2 ± 12.2	35.8 ± 3.8
TB(îmol/L)	7.8 ± 2.2	12.6 ± 4.3	331.2 ± 70.0
HBV-DNA(log10 IU/mL)	NA	5.5 ± 1.9	4.2 ± 1.1

Bioinformatics analysis

To understand the potential roles of these differentialy expressed miRNAs, TargetScan (http://www.targetscan.org/vert_71/) was engaged to predict their target genes. The obtained target genes were further annotated by The Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/list.jsp).

According to the GO enrichment analyses, 908 GOs were modulated by the down-regulated genes, whereas 771 GOs were modulated by the up-regulated genes. An integrative GO analysis of these target genes were showed in figure 3A. The top 10 GO categories involved in genes targeted by the down-regulated miRNAs including positive regulation of gene expression, regulation of transcription from RNA polymerase II promoter, positive regulation of DNA-dependent transcription etc. (Figure 3B). while the top 10 GO categories involved in genes targeted by the up-regulated miRNAs including regulation of transcription, regulation of transcription from RNA polymerase II promoter, positive regulation of gene expression, etc. (Figure 3C).

KEGG pathway analyses showed that the target genes of up-regulated and down-regulated miRNAs were implicated in 60 and 65 pathways respectively. An integrative KEGG pathway analysis of these target genes were showed in figure 4A. The top 12 pathways involved in genes targeted by the down-regulated miRNAs are showed in figure 4B, while the top 12 pathways involved in genes targeted by the down-regulated miRNAs are presented in figure 4C.

Establishment of miRNA-gene/gene-pathway networks

Based on target prediction, the network between the top 100 targets of the top 3 up-and down-regulated miRNAs was visualize in figure 5A. Pathway enrichment

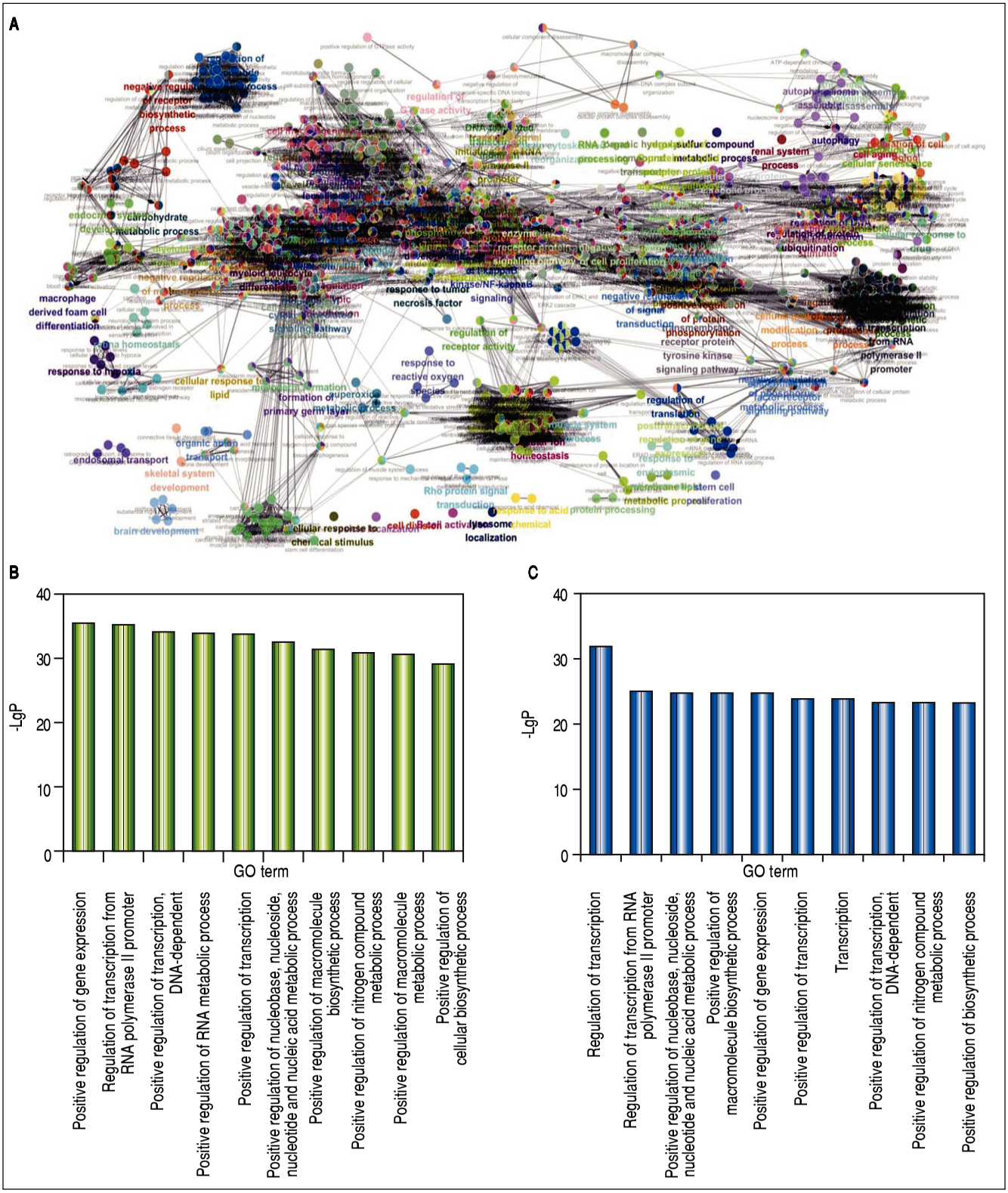

Figure 3. *GO analysis for predicted target genes.* ***A.*** *An integrative GO term analysis of the target genes, each node represents a GO term, each color represents a functional group, terms are connected based on shared genes, the node size represents the term enrichment significance.* ***B.*** *The top 10 GO terms of genes targeted by the down-regulated miRNAs.* ***C.*** *The top 10 GO terms of genes targeted by the up-regulated miRNAs.*

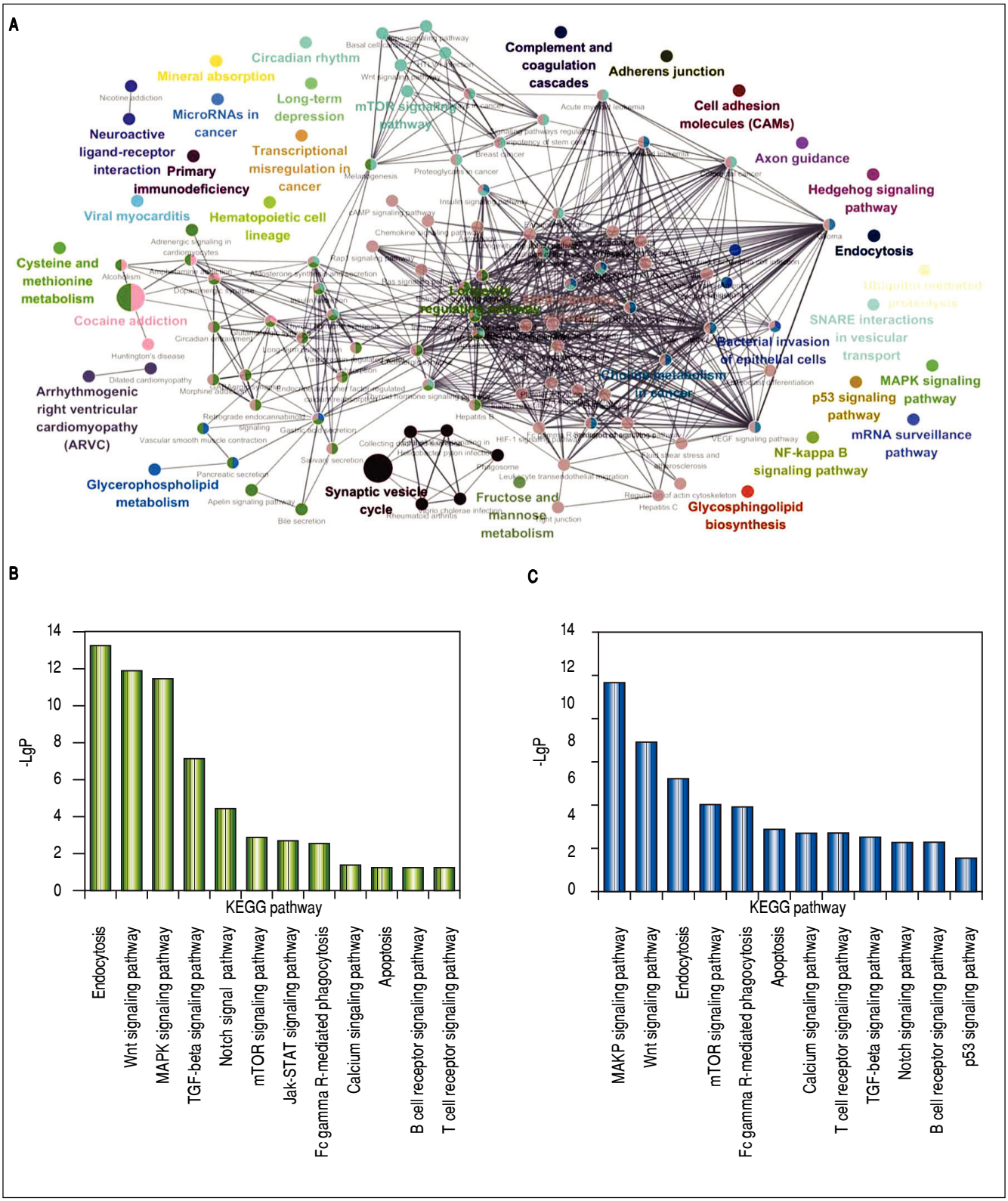

Figure 4. *KEGG pathway analysis for predicted target genes.* ***A.*** *An integrative KEGG pathway analysis of the target genes, each node represents a KEGG pathway, each color represents a functional group, pathways are connected based on shared genes, the node size represents the pathway enrichment significance.* ***B.*** *The top 12 pathways targeted by the down-regulated miRNAs.* ***C.*** *The top 12 pathways targeted by the up-regulated miRNAs.*

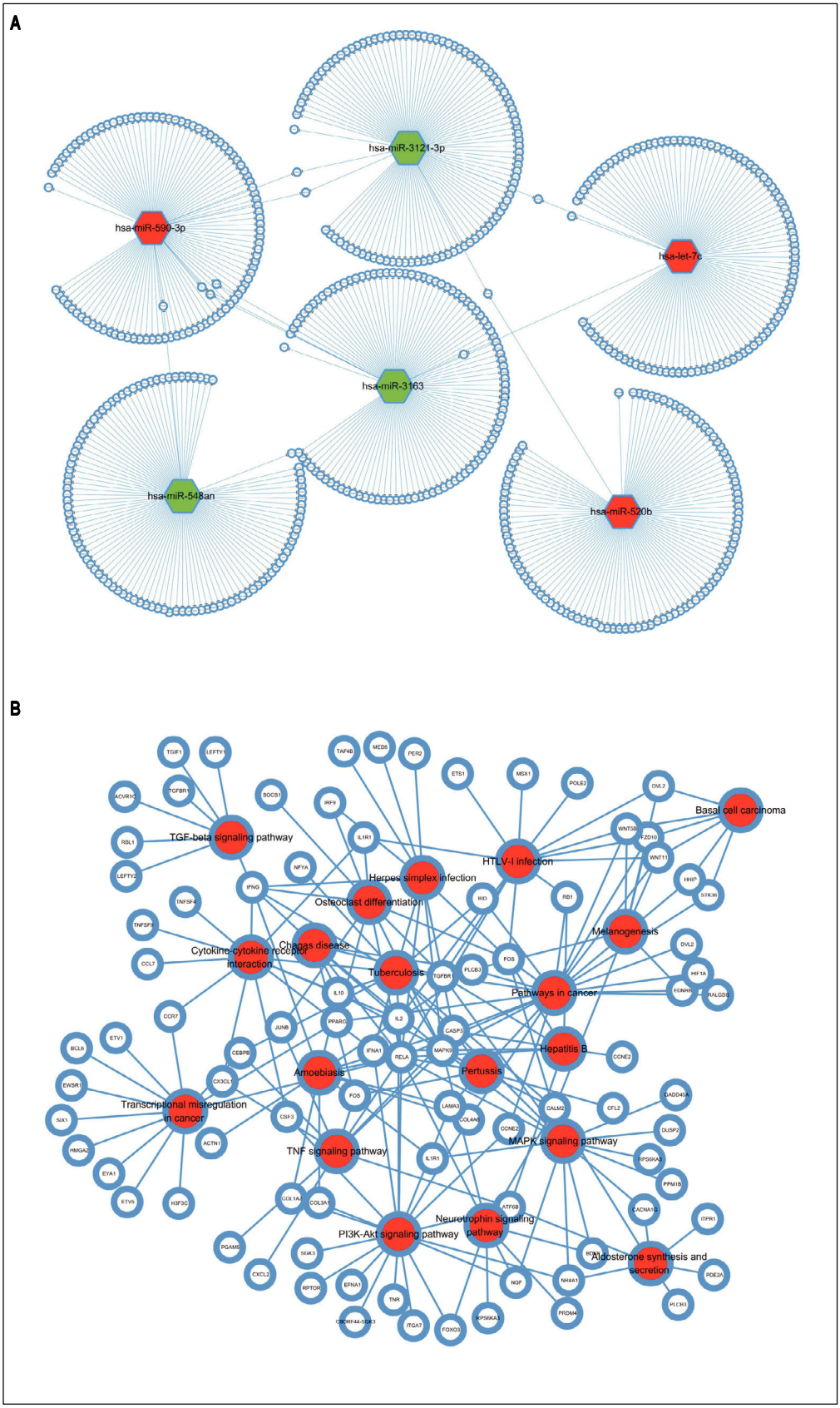

Figure 5. *Network of miRNA-gene/gene-pathway.* ***A.*** *miRNA-target gene network.* ***B.*** *Target gene-pathway network. White circle node with blue border represents target genes. Oxygen nodes represent miRNAs (green node indicate down-regulated, red node indicate up-regulated) and oval nodes represent mRNAs. Red node represents target genes involved pathways.*

analysis of the target genes using DAVID database was displayed in figure 5B. According to analysis, CCNE2, FOS, CASP3, IFNA1, RELA, TGFBR1, ATF6B, MAPK9 and RB1 were implicated in hepatitis B.

DISCUSSION

An increasing number of studies have revealed the roles of miRNAs in virus infection, including HBV infection. The interaction between host immune response and viral replication determines the clinical outcome of HBV infection. Given that PBMC is an integral part of immune system, we investigated the miRNA expression profile in PBMCs to explore the potential role of miRNA in the pathogenesis of CHB.

In the current study, we analyzed the miRNA expression profiles in PBMCs of normal volunteers and patients with CHB/ACLF. Among thousands of aberrant expressed miRNAs, 45 up-regulated and 62 down-regulated miRNAs were found with fold expression change ≥ 1.5 in patients with CHB/ACHBLF compared with healthy donors. The results suggested that these miRNAs may be related to the immune response of host and progression of HBV infection.

Significance test of target genes by Gene Ontology revealed that gene expression, transcription, metabolic process and biosynthetic process are primary biological processes regulated by differentially expressed miRNAs; KEGG annotation demonstrated that these miRNAs mainly target pathways like MAPK signaling pathway, Endocytosis, Wnt signaling pathway, TGF-beta signaling pathway, Nocth signaling pathway and mTOR signaling pathway, etc. These signaling pathways have been implicated in the pathogenesis or the development of CHB. For example, it has been proven that HBV, through X protein, can utilize MAPK signaling pathway to deregulate cell cycle and promote cell survival, even hepatocarcinogenesis.[16-18] Besides, it has also been reported that HBV surface antigen can selectively inhibits IL-12 production in monocytes/macrophages by interfering the JNK-MAPK signaling pathway and thus evades immunity and lead to HBV persistence.[19] In immortalized human primary hepatocytes, clathrin-dependent endocytosis has been identified as a way of HBV entry.[20] Wnt signaling plays critical roles in diverse human biology, including embryogenesis, organogenesis, and maintaining tissue homeostasis.[21] In liver, it regulates various cellular processes, including proliferation, differentiation, survival and others.[21]

By pathway analysis, CCNE2, FOS, CASP3, IFNA1, RELA, TGFBR1, ATF6B, MAPK9 and RB1 are involved in hepatitis B. FOS, CASP3, IFNA1, RELA, TGFBR1, MAPK9 and RB1 have been reported play roles in HBV infection or CHB progression.[22-28] However, the function of CCNE2 and ATF6B in HBV infection remains poorly understood. CCNE2 encodes cyclin E2, which plays a role in cell cycle G1/S transition and has been implicated in mouse hepatocyte proliferation.[29] ATF6B is a transcription factor in the unfolded protein response during endoplasmic reticulum stress.[30,31] Study has revealed that ATF6 beta is a host cellular target of the Toxoplasma gondii virulence factor.[32]

In summary, we identified that 107 miRNAs were differentially expressed in PBMCs from patients with CHB/ACLF. Bioinformatics analysis revealed that the target genes of these miRNAs were implicated in multiple biological processes and signaling pathways, especially gene expression regulation. The current study provides alternative insights into the pathogenesis of HBV-related liver disease, and the potential mechanisms deserve further validation.

ABBREVIATIONS

- **ACLF:** acute-on-chronic liver failure.
- **CHB:** chronic hepatitis B.
- **DAVID:** The Database for Annotation, Visualization and Integrated Discovery.
- **GO:** gene ontology.
- **HBV:** hepatitis B virus.
- **IFN:** interferon.
- **KEGG:** Kyoto Encyclopedia of Genes and Genomes.
- **miRNA:** microRNA.
- **PBMC:** peripheral blood mononuclear cell.

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CONFLICTS OF INTEREST

There were no financial disclosures from any authors.

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