

Original Article

Screening and bioinformatic analysis of microRNA-associated immune clearance in patients with chronic hepatitis B

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Abstract: Objective: This study aimed to perform screening and bioinformatic analysis of microRNA (miRNA) molecules associated with immune clearance in chronic hepatitis B (CHB) patients. Methods: Peripheral blood mononuclear cells (PBMCs) of CHB patients and healthy individuals were collected and detected by microarray. The target genes of differentially expressed miRNA molecules were predicted using three databases. Their molecular pathways and functions were analyzed by bioinformatics methods. Results: Compared with healthy individuals, 52 differentially expressed miRNA molecules were found in PBMCs of CHB patients, of which 33 were up-regulated and 19 were down-regulated. A total of 354 target genes were predicted in up-regulated miRNA molecules, and 1935 target genes were predicted in down-regulated miRNA molecules. MiRNA-mRNA network analysis showed that some target genes might be regulated, and constituted complex molecular networks with hsa-miR-520d-5p, hsa-miR-106a-5p, hsa-miR-30a-5p, and hsa-miR-29b-3p. Gene ontology and pathway analyses showed that several molecular pathways might be affected by up- or down-regulated miRNA molecules. Conclusion: Abnormal expression of multiple miRNA molecules in PBMCs of CHB patients might be involved in immune clearance pathogenesis through the regulation of multiple molecular pathways and target genes.

Keywords: Chronic hepatitis B, microRNA, immune clearance

Introduction

Chronic hepatitis B virus (HBV) infection is one of the major infectious diseases causing serious harm to human health at present. Immune dysfunction of the host might play a key role in the pathogenesis and prognosis of chronic HBV infection [1]. The natural history of perinatally acquired chronic HBV infection often involves a long period of immune tolerance. Spontaneous or treatment-induced chronic HBV infection enters the phase of immune clearance and develops into chronic hepatitis B (CHB) [2]. The reasons for this transition from immune tolerance to immune clearance may be related to the enhancement of specific immune function or virus variation. However, the accurate molecular mechanism remains unclear [3].

MicroRNAs (miRNAs) are small non-coding RNAs that play critical roles in the regulation of many biological processes at the post-transcriptional level [4].

The abnormal expression levels of miRNAs have been revealed in various diseases, such as cancer, cardiovascular diseases, and viral infections, including HBV [5-7]. Some miRNAs (e.g., miRNA-155, -146, and -181a) were recently found to play important roles in the development and function of the immune system and immune regulation [8, 9]. Studies have shown that certain miRNA molecules are involved in the pathogenesis of chronic HBV infection. MiRNA molecules in serum can be used as new potential molecular markers to predict liver injury [10, 11]. However, the expression patterns of immune-related miRNAs in CHB patients and its regulatory mechanism have not been reported. In this study, miRNA expression of peripheral blood mononuclear cells (PBMCs) in patients with CHB was detected by miRNA microarray. Immune-related miRNA molecules were screened, and target genes were predicted and analyzed using bioinformatic methods.

Table 1. Baseline data of groups detected by microarray

	CHB	HC
Sex (m/f)	3/9	2/7
Age (y)	33±8	30±6
ALT (U/L)	216.5±273.5	24.1±6.2
HBV DNA (Log ¹⁰ copies/mL)	5.7±1.5	-

Methods

Subjects

Blood samples were collected from 12 CHB patients at the Taizhou People's Hospital from 2011 to 2012. The healthy controls consisted of nine cases. The baseline data of the two groups are shown in **Table 1**. Written informed consent was obtained from all subjects. The experimental protocol was approved by the ethical commission of Taizhou People's Hospital. The diagnostic criteria were based on the 2010 Chronic Hepatitis B Prevention Guide of China [12]. All patients were negative for antibodies against hepatitis A, C, D, and E viruses, as well as human immunodeficiency virus. All patients with history and clinical features of drug-induced liver injury, alcoholic hepatitis, and steatohepatitis, as well as those treated with nucleotide/nucleoside analogues and antiviral or immunomodulatory drugs in the previous six months, were excluded.

PBMC separation, RNA extraction, and miRNA microarray

1. PBMC separation. PBMCs were separated by Ficoll-Hypaque gradient centrifugation. PBMCs (5×10^6) were collected, added with 1 mL of TRIzol (Invitrogen), and frozen at -80°C until examined. The PBMCs of three subjects from the same group were pooled, and three or four pools were analyzed in the microarray groups.

2. RNA extraction and labeling. Total RNA was isolated using TRIzol (Invitrogen) and miRNeasy mini kits (QIAGEN) according to the manufacturer's instructions. All RNA species, including miRNAs, were efficiently recovered. RNA quality and quantity were measured using a nanodrop spectrophotometer (ND-1000, Nanodrop Technologies). RNA integrity was determined by gel electrophoresis. MiRNA labeling was performed using a miRCURYTM Hy3TM/Hy5TM Power labeling kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions.

3. Array hybridization. The Hy3TM-labeled samples were hybridized using a miRCURYTM LNA array (v.18.0) kit (Exiqon) according to the manufacturer's instructions. In brief, a mixture of 25 μL of Hy3TM-labeled samples and 25 μL of hybridization buffer was denatured for 2 min at 95°C , incubated on ice for 2 min, and then hybridized to the microarray for 16 h to 20 h at 56°C in a 12-bay hybridization system (Hybridization System, Nimblegen Systems, Inc., Madison, WI, USA). This system provides an active mixing action and constant incubation temperature to improve hybridization uniformity and enhance the signal. After hybridization, the slides were washed several times using wash buffer (Exiqon), and dried by centrifugation for 5 min at 400 rpm. The slides were scanned using an Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA).

4. Data analysis. The scanned images were imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. The replicated miRNA molecules were averaged, and miRNA molecules with intensities ≥ 50 in all samples were selected for calculation of the normalization factor. The expressed data were normalized using median normalization. Significantly differentially expressed miRNAs were identified by volcano plot filtering. Hierarchical clustering was performed using MEV software (v4.6, TIGR). Significant upward or downward trends were observed when the standard value of the experimental group was 1.5 times higher or 0.67 times lower, respectively, than that of the control group ($P < 0.05$).

Prediction of target gene of up- or down-regulated miRNAs

Possible target genes of up- or down-regulated miRNAs were predicted by Target Scan (<http://www.targetscan.org>), miRbase (<http://www.mirbase.org/>), and miRadna (<http://www.microna.org>). The intersection of these three databases was obtained.

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses of possible up- or down-regulated target genes

MiRNA targets were subjected to GO (<http://www.geneontology.org>) analysis to uncover the miRNA-gene regulatory network based on the biological processes and molecular functions.

Immune miRNA of chronic hepatitis B

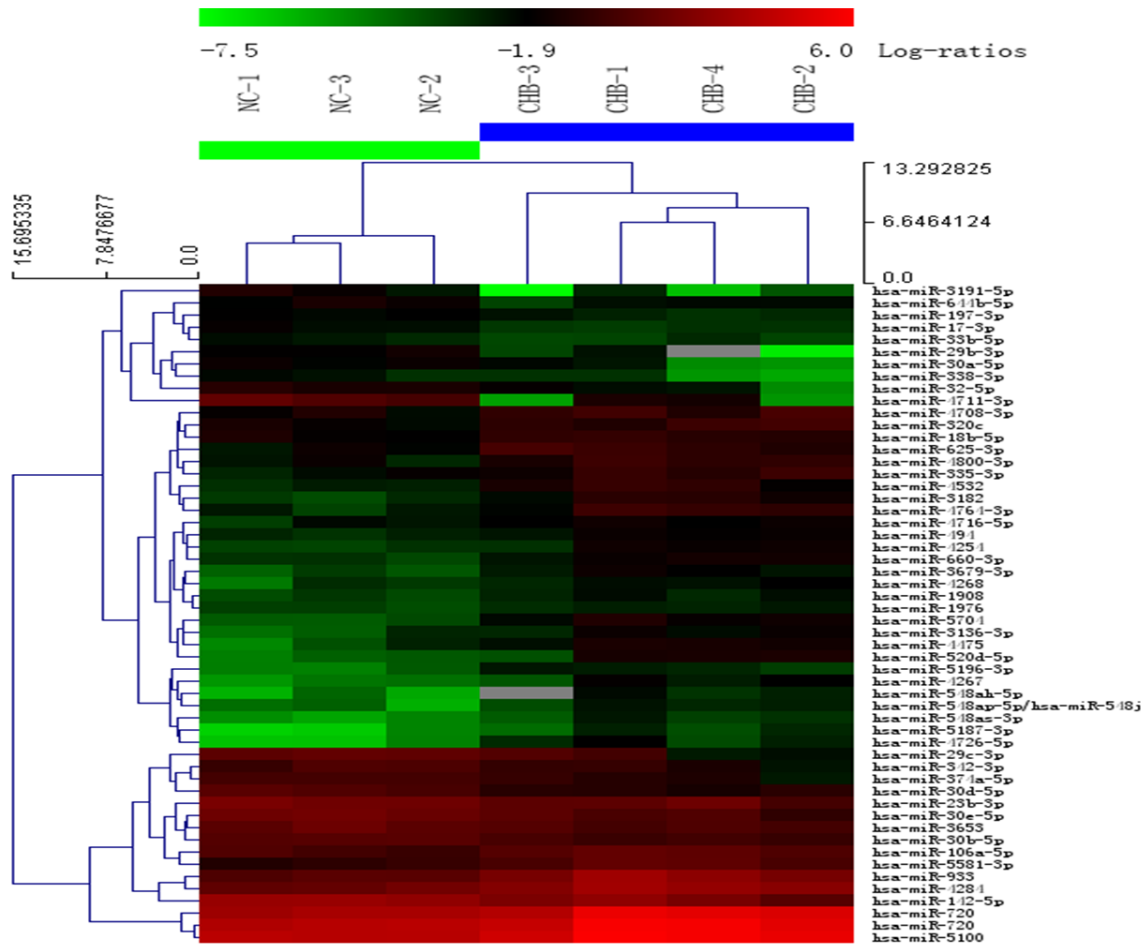


Figure 1. Hierarchical cluster analysis using total miRNA of PBMC in CHB and HC. CHB, chronic hepatitis B. NC, normal control. Note: 1. Red represents that the upregulation of miRNA; Green represents a downregulation of miRNA. 2. The PBMCs of three subjects from the same group were pooled and four or three pools were analyzed.

Enrichment provided a measure of the significance of the function. Pathway analysis was used to determine the significant pathway of the differential genes according to KEGG (<http://www.genome.jp>). Fisher's exact and χ^2 test were used to select the significant pathway. The false discovery rate was calculated to correct the P value. Statistical significance was set at $P < 0.05$.

MiRNA gene regulatory network between different miRNA molecules and target genes

Based on the Targetscan database, target genes were predicted. Moreover, the different expression of all target genes corresponding to miRNA molecules was obtained. The network map of miRNAs and their corresponding target genes were constructed according to the relationship between the miRNA and target gene.

Statistical analysis

Data are expressed as mean \pm standard deviation. The t-test was used for comparison between two groups. Data analysis was performed using SPSS17.0 statistical software (SPSS, Inc.). Statistical significance was set at $P < 0.05$.

Results

Expression of miRNAs in PBMCs of CHB patients

MiRNA microarray was used to detect miRNA expression in PBMCs of CHB patients. Hierarchical cluster analysis was performed to analyze the resulting data (Figure 1). Compared with the controls, 33 significantly up-regulated and 19 significantly down-regulated miRNAs

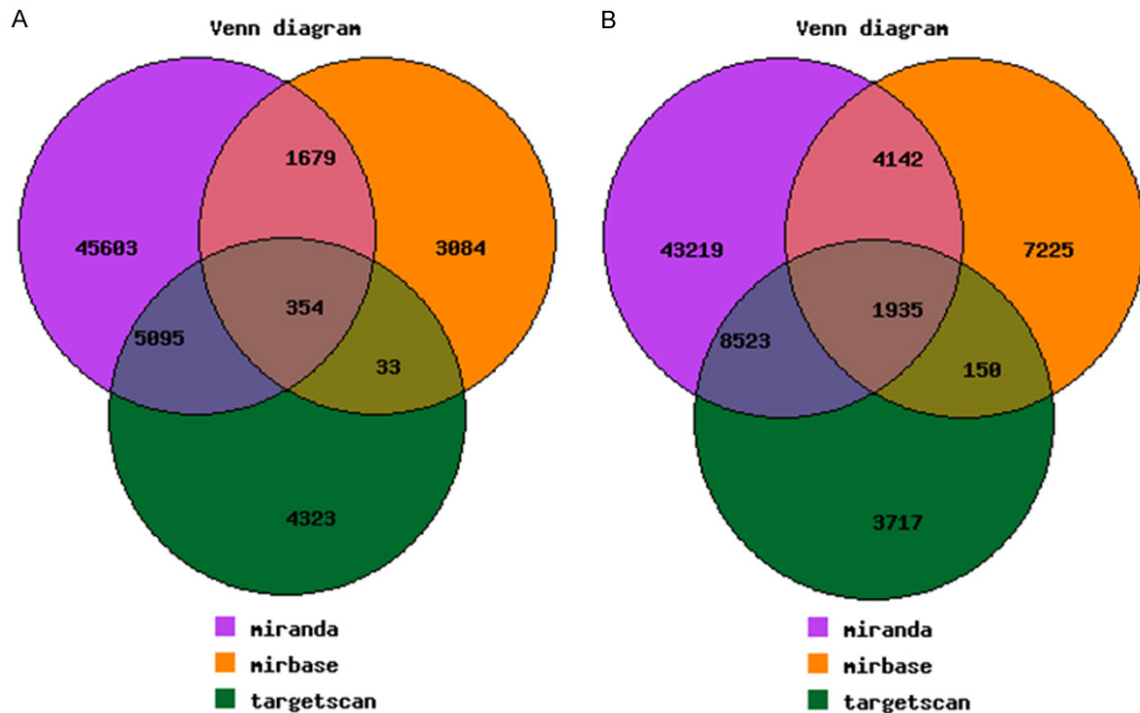


Figure 2. The intersection graphs of miRNAs target genes. A: Up-regulation, B: Down-regulation.

were identified in PBMCs of CHB patients. Among 33 up-regulated miRNAs, five miRNAs (i.e., hsa-miR-4726-5p, hsa-miR-4267, hsa-miR-520d-5p, hsa-miR-548ah-5p, and hsa-miR-5187-3p) increased by more than five times. Among 19 down-regulated miRNAs, two miRNAs (i.e., hsa-miR-4711-3p and hsa-miR-3191-5p) decreased by more than five times.

Prediction of target genes of up- or down-regulated miRNAs

The intersections of up- or down-regulated miRNA target genes were analyzed by TargetScan, miRbase, and miRadna. The number of up-regulated target genes was 354. The number of down-regulated target genes was 1935 (**Figure 2**).

GO or pathway analysis of target genes of up- or down-regulated miRNAs

The molecular functions of up- or down-regulated miRNA target genes were analyzed by GO online. The results show that the molecular functions of target genes of up-regulated miRNAs were enriched in apolipoprotein receptor activity, nucleoside two phosphatase activity, mitogen-activated protein kinase (MAPK) activ-

ity, and low density lipoprotein receptor activity. The molecular functions of target genes of down-regulated miRNAs were concentrated in platelet-derived growth factor activity, platelet-derived growth factor receptor binding activity, activin binding activity, uridylylase kinase activity, and small molecules in nuclear GTP binding protein activity (**Figure 3**).

Pathway analysis of KEGG showed that the molecular pathways regulating the up-regulation of miRNA molecules mainly included the MAPK signal pathway, cancer-related pathways, Notch signaling pathway, stimulation of shape forming factor signaling pathway, and Wnt pathway. The molecular pathways regulating the down-regulation of miRNA molecules mainly included the protein digestion pathway, ECM receptor binding and uptake pathways, local adhesion pathways, PI3K-Akt signaling pathway, and gap interaction approach (**Figure 4**).

Analysis of miRNA-GO-network of up- and down-regulated miRNA molecules

Enrichment analysis was used to investigate the biological processes of GO and miRNAs. The chart of miRNA-GO-network analysis

Immune miRNA of chronic hepatitis B

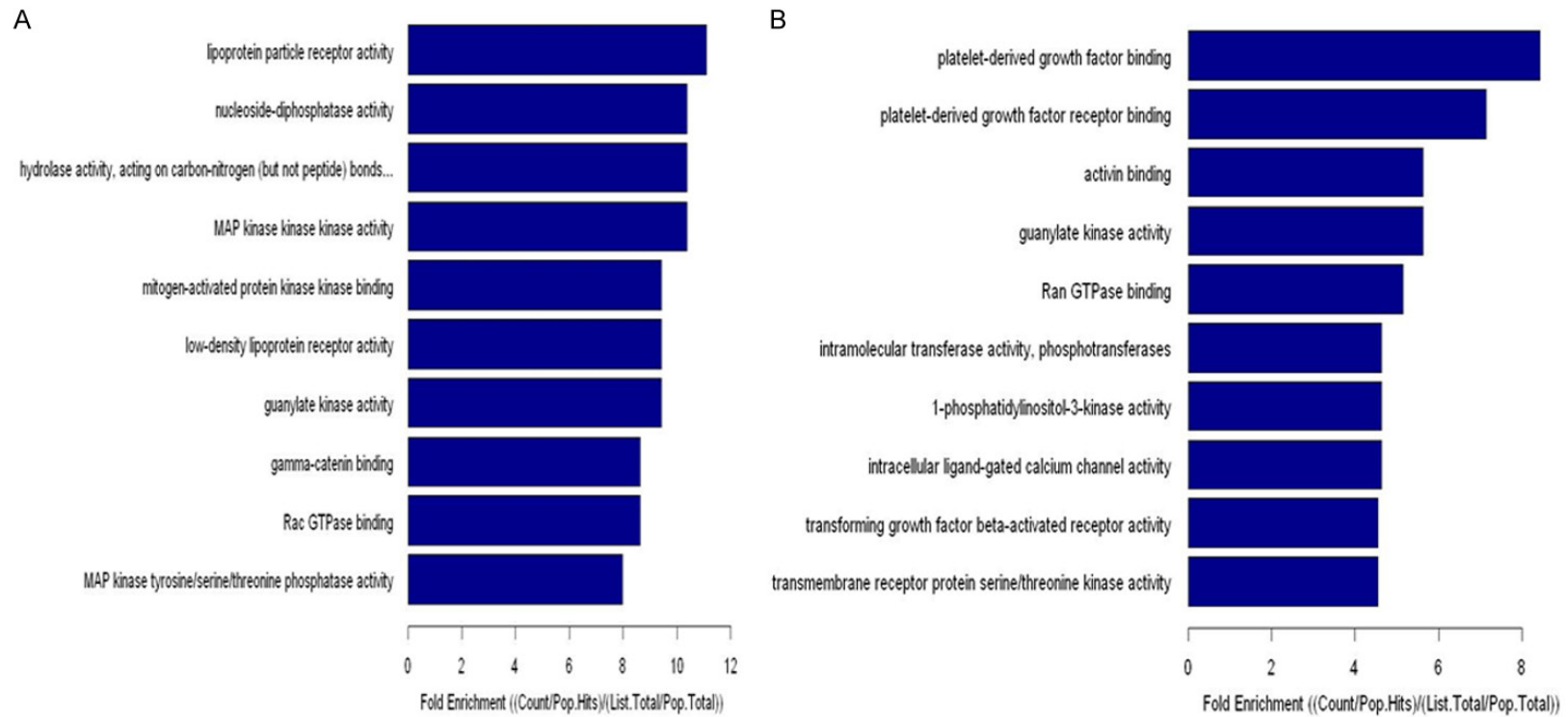


Figure 3. The GO analysis maps of miRNAs target genes. A: Up-regulation, B: Down-regulation.

Immune miRNA of chronic hepatitis B

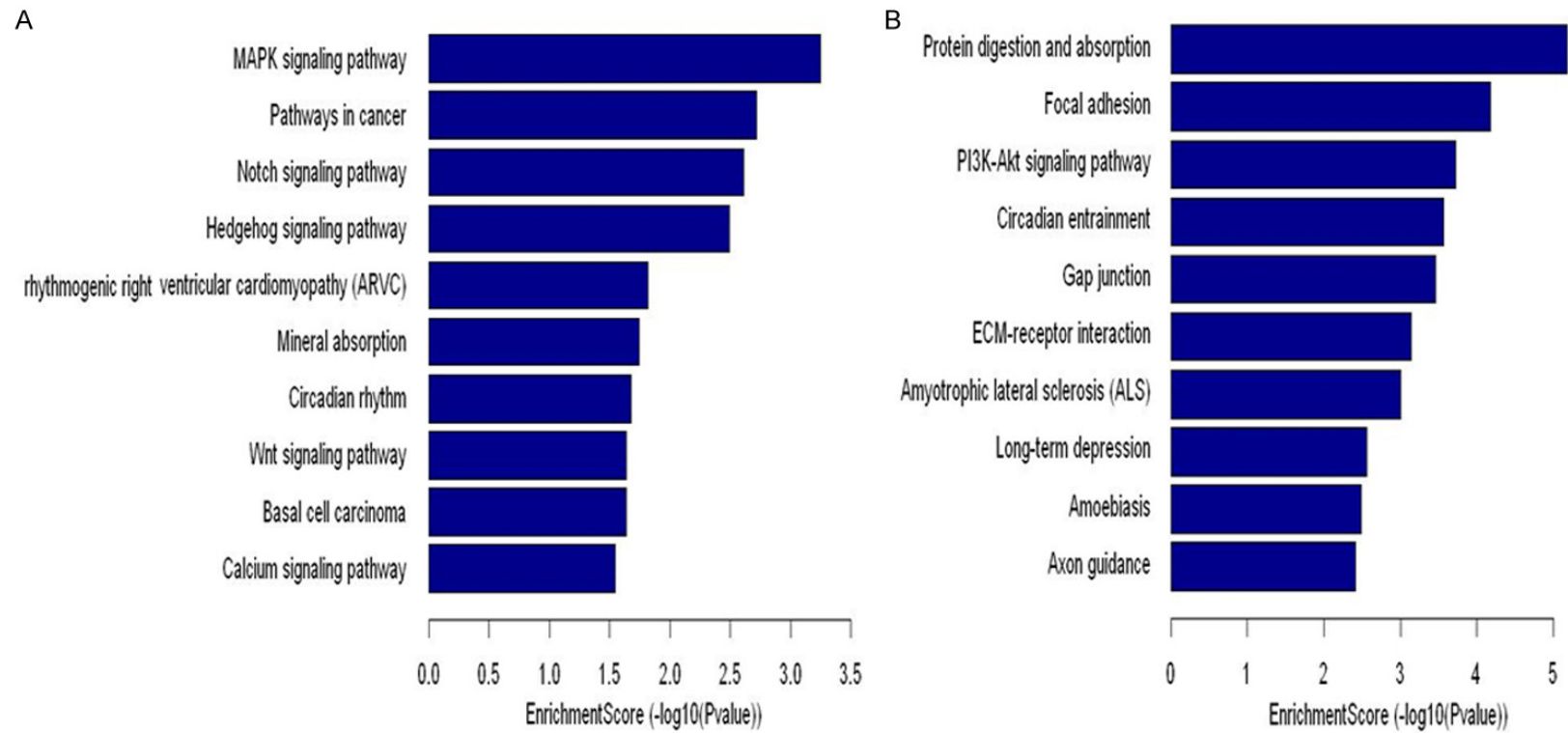


Figure 4. The Pathway analysis maps of miRNAs target genes. A: Up-regulation, B: Down-regulation.

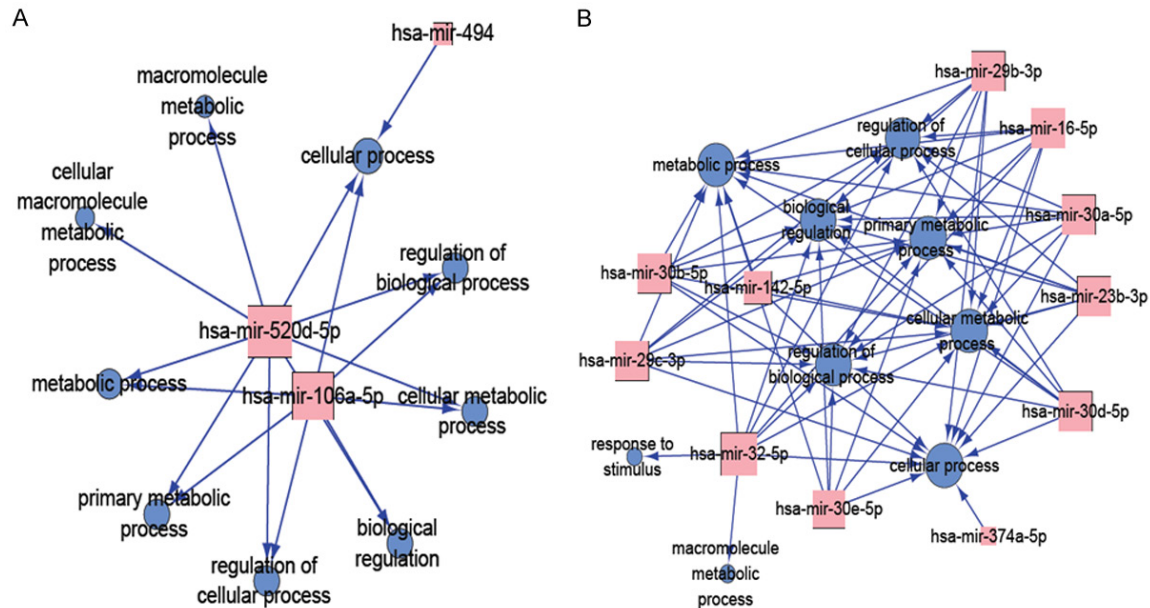


Figure 5. miRNA-GO-network of different miRNAs. A: Up-regulation, B: Down-regulation.

results is formed by gene-set associations. The overlap number of >3 and overlap coefficient >0.5 were used as the association strength index. The results show that hsa-miR-520d-5p, hsa-miR-106a-5p, and other up-regulated miRNAs centralized the regulation of cell metabolism and macromolecule metabolic process. The down-regulated molecules hsa-miR-29 and hsa-miR-30 were involved in cell metabolism, macromolecule metabolic process, and biological process (**Figure 5**).

Analysis of miRNA-mRNA network of up- and down-regulated miRNA molecules

The miRNA-gene-network was constructed based on the relationship between up- and down-regulated miRNAs and their target genes. The results show that the up-regulated miRNA molecules (hsa-miR-520d-5p, hsa-miR-106a-5p, etc.) and down-regulated miRNA molecules (hsa-miR-30a-5p, hsa-miR-29b-3p, etc.) both regulated a large number of target genes and constituted intensive molecular networks. Among them, not only there are genes related to cytokines played important roles in immune function, such as IL-1a, IL-28Ra, IFN-aR2, etc., but also important gene associated with immune signaling pathways, such as STAT-3, STAT-4, SOCS-1 molecules in JAK-STAT signaling pathway associated with interferon and cytokine; TNFRSF1, TRAM, Bcl-3, etc. in NF-kB

signal pathway and Trim-3, Trim-8, Trim-37 molecules of TRIM protein family.

Discussion

The enhancement of specific immune function (mainly specific cytotoxic T cells, Th1, and Treg cell functions) may be related to the transition from immune tolerance to immune clearance on the natural history of perinatally acquired chronic HBV infection [3, 13]. Studies showed that various miRNA molecules, such as miRNA-146a, 150, and 155, play an important role in the differentiation and maturation of immune cells [14, 15]. Chen et al. [16] reported 17 miRNA molecules (five up-regulated and 12 down-regulated) in PBMCs of acute chronic liver failure patients by miRNA microarray analysis. Winther [17] showed 16 differentially expressed miRNAs, including miRNA-122, -192, and -455, in the plasma of patients with HBeAg-positive CHB in children. In the present study, the results show the abnormal expression of many types of miRNA molecules in PBMCs of CHB patients. A total of 33 up-regulated and 19 down-regulated miRNA molecules were included. The expression patterns in this study were similar to those reported by Chen and Winther. However, the specific molecules differed compared with those in their reports. This difference may be related to patient selection, severity of the disease, different types of samples,

and application of the chip. Some studies have indicated that the expression of two miRNA molecules, namely, hsa-miR-548ah-5p and hsa-miR-29b-3p, is related to the pathogenesis of CHB [18, 19]. Hsa-miR-548ah-5p and hsa-miR-29b-3p regulate immune function by regulating the expression of IFN- λ 1 and IFN- γ , respectively. The functions of hsa-miR-4711-3p, hsa-miR-3191-5p, hsa-miR-5704, and other molecules have not been reported and require further research.

Functional enrichment analysis showed that the molecular functions of target genes regulated by up-regulated miRNA molecules were enriched in mitogen-activated apolipoprotein receptor activity, nucleoside two phosphatase activity, MAPK activity, low density lipoprotein receptor activity, and silk protein kinase activity. The molecular functions of target genes regulated by down-regulated miRNA molecules were concentrated in platelet-derived growth factor activity, platelet-derived growth factor receptor binding activity, activin binding activity, uridylate kinase activity, and small molecules in nuclear GTP binding protein activity. Further analysis of the signal transduction pathway of target genes revealed that the molecular pathways regulated by up-regulated miRNA molecules mainly included the MAPK signal pathway, Notch pathway, and Wnt pathway. The molecular pathways of target genes regulated by down-regulated miRNA included the molecular PI3K-Akt signal pathway, as well as the major gap binding and ECM receptor interaction pathway. These molecular pathways were involved in cell responses and physiological and pathological processes, which are worthy of further investigation regarding their role in the pathogenesis of chronic HBV infection.

Research showed that miRNA molecules mainly play their regulatory functions at post-transcription. The regulatory effects of miRNA molecules are pleiotropic. The biological process of miRNAs and GO enrichment analysis showed that up-regulated hsa-miR-106a-5p and hsa-miR-520d-5p, as well as down-regulated hsa-miR-29b-3p, hsa-miR-30a-5p, and other molecules, were involved in the regulation of cell metabolism and macromolecule metabolic process. The results of miRNA-gene-network analysis showed that the target gene, up-regulated molecules (i.e., hsa-miR-106a-5p and hsa-miR-520d-5p), down-regulated molecules (i.e., hsa-

miR-29b-3p and hsa-miR-30a-5p), and other molecules comprised an intensive network. Some immune-related target genes were associated with the pathogenesis of CHB. Hsa-miR-520d-5p can regulate Bcl-3, signal transducer and activator of transcription (STAT) 4, and pDC-1 gene [20]. Hsa-miR-106a-5p regulates the expression of TRIM-3, TRIM-8, and STAT3 genes. Hsa-miR-29b-3p regulates genes including interferon-inducible protein-30, interferon-stimulated gene-20, and TNFRSF1A [21]. Hsa-miR-30a-5p targets genes including IL-1, IL-28R, suppressor of cytokine signaling-1, and IFN- α 2. The aforementioned results suggested that miRNA expression in PBMCs of CHB patients might regulate the function of numerous target genes, which play important roles in the pathogenesis of CHB.

In conclusion, this study showed that multiple abnormal expression of miRNA molecules in PBMCs of CHB patients was involved in immune clearance mechanisms of CHB possibly through the regulation of multiple molecular pathways and target genes.

Disclosure of conflict of interest

None.

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