Int J Clin Exp Med 2018;11(10):10475-10486
www.ijcem.com /ISSN:1940-5901/IJCEM0072391

Original Article

Immune clearance-associated microRNAs in circulating Microvesicles from chronic hepatitis B patients

Fan Yang[1*], Yao Wang[2*], Juan Xue[3*], Xin Cai[4], Lei Luo[4], Hui-Kun Wu[1], Wei Ni[1], Jing-Jing Huang[1], Xiao-Dong Li[1]

[1]*Department of Hepatology, Hubei Provincial Hospital of Chinese Medicine, Wuhan 430061, PR China;* [2]*Department of Infectious Diseases, Renmin Hospital of Wuhan University, Wuhan 430060, PR China;* [3]*Department of Gastroenterology, Hubei Province Hospital of Integrated Traditional Chinese and Western Medicine, Wuhan 430015, PR China;* [4]*School of First Clinical Medicine, Hubei University of Chinese Medicine, Wuhan 430061, PR China.* [*]*Equal contributors.*

Received June 29, 2017; Accepted May 24, 2018; Epub October 15, 2018; Published October 30, 2018

Abstract: Objective: This study aimed to perform screening and bioinformatic analysis of microRNA (miRNA) molecules enriched in microvesicles (MVs) associated with immune clearance in chronic hepatitis B (CHB) patients. Methods: MVs of CHB patients and healthy individuals were collected and detected by microarray. Then we respectively selected 3 up-regulated and 3 down-regulated miRNAs that with the biggest fold change and P < 0.05 as our research objects. The target genes of differentially expressed miRNA molecules were predicted using two databases. Their molecular pathways and functions were analyzed by bioinformatics methods. Results: Compared with healthy individuals, 221 differentially expressed miRNA molecules were found in MVs of CHB patients, of which 101 were up-regulated and 120 were down-regulated. Then we selected the biggest 15 fold change and P < 0.05 miRNAs as our research objects. Compared with the MVs from normal people, miR-4498, miR-3065-3p and miR-370-5p were obviously increased (P < 0.01), and miR-148a-5p, miR-222-5p and miR-223-5p were obviously decreased (P < 0.01) in the MVs from CHB patients. A total of 1289 target genes were predicted in up-regulated and down-regulated miRNA molecules. MiRNA-mRNA network analysis showed that some target genes might be regulated, and constituted complex molecular networks with hsa-miR-4498, hsa-miR-106a-5p, hsa-miR-512-3p and hsa-miR-429, et al. 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 MVs of CHB patients might be involved in immune clearance pathogenesis through the regulation of multiple molecular pathways and target genes.

Keywords: Chronic hepatitis B, microvesicles, microRNA, immune clearance

Introduction

Hepatitis B virus (HBV) is a hepatotropic DNA virus that primarily infects hepatocytes, and then cause immune dysfunction of the host, which plays a crucial role in the process of pathogenesis and prognosis [1, 2]. According to the statistics, approximately 240 million people are chronically infected with HBV worldwide [3]. Moreover, the hepatitis B surface antigen (HBsAg) positive rate in the general population was reported to be nearly about 10% in China [4]. If the Chronic HBV infection cannot get timely treatment, it would lead to advanced stage liver diseases, including cirrhosis and hepatocellular carcinoma (HCC).

MicroRNAs (miRNAs), small single-stranded non-coding RNAs (19-23 nt), are able to silence endogenous messenger RNA (mRNA) transcripts. Therefore, miRNAs could modulate gene expression by degrading or inhibiting mRNAs and suppress protein translations at post-transcriptional level [5]. Given the important roles of miRNAs in cell energy production, protein synthesis, proliferation, differentiation and apoptosis, it should come as no surprise that dysregulated miRNA play important roles in a variety of diseases, including chronic HBV infection and HCC genesis [6].

Microvesicles (MVs) is a cell-derived vesicle, defined as 100-1000 nm in size, which is

Table 1. Baseline data of groups detected by microarray

	CHB (n = 33)	NC (n = 33)
Sex (m/f)	10/23	14/19
Age (y)	48.3 ± 12.1	46.7 ± 10.9
HBsAg (qualitative)	+	-
ALT (U/L)	197.5 ± 73.5	22.7 ± 6.8
AST (U/L)	213.5 ± 84.5	26.3 ± 18.7
HBV DNA (Log^{10} copies/mL)	7.3 ± 1.2	-

formed from the outward-budding of the plasmamembrane and subsequent shedding out from the cells [7]. MVs are released by various liver related cell types, including hepatocyte, hepatic stellate cell (HSC) and macrophages monocytes [8]. And the cargo of MVs consists of bioactive molecules, including proteins, lipids and nucleic acids (RNA, microRNA, DNA) that derived from the originate cell [8]. So, the function of MVs is dependent on the cargo they carry [9].

However, the pathogenesis of HBV infection remains poorly understood and the expression patterns of immune-related miRNAs in CHB patients and its regulatory mechanism have not been reported. So in this study, miRNA expression of MVs in CHB patients was detected by miRNA microarray. Immune-related miRNA molecules were screened, and target genes were predicted and analyzed using bioinformatic methods.

Methods

Subjects

Thirty-three blood samples for CHB patients were collected from Hubei Provincial Hospital of Chinese Medicine from 2015 to 2017. The healthy controls consisted of 33 cases. Three of the CHB patients and 3 of healthy controls were selected to detect by microarray. The other 30 CHB patients and 30 of healthy controls were selected to verified the differentially expressed microRNAs. 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 2015 Chronic Hepatitis B Prevention Guide of China [10]. 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 [11]. The patients with chronic hepatitis B in immune clearance referred that the surface antigen was positive and HBV-DNA was elevated with abnormal liver function [12]. The hepatitis B patients selected in this study meet the above requirements.

Blood sampling and plasma preparation

Blood, 4 ml volume from each participant, gathered into EDTA-K2 containing tubes was brought to laboratory for processing within 12 h. Specimens were drawn at 8 o'clock in the morning from participants with an overnight fast. The whole blood taken from every subject was centrifuged at 1500 × g for 10 min at 4°C to be separated into plasma and cellular fractions. Then upper plasma was moved into a clean tube for further conduction.

MVs isolation

Obtained plasma was centrifuged at 2000 × g for 30 min at 4°C to remove remaining cell fractions and debris. Then the supernatant gained was transferred to a fresh tube and centrifuged at 20,000 × g for 2 h at 4°C [13]. MVs accumulated in the bottom of the tube and upper liquid was discarded. Most of MVs sediments were resuspended in lysate and then frozen at -80°C for the next step. A few of MVs precipitates were fixed in PBS with 2.5% (w/v) glutaraldehyde, dehydrated, embedded, sliced, and stained. The ultrathin sections were examined in a transmission electron microscope (Tecnai G2 12, FEI).

RNA extraction

Total RNA was isolated using TRIzol (Invitrogen) and purified with RNeasy mini kit (QIAGEN) according to manufacturer's instructions. RNA quality and quantity was measured by using nanodrop spectrophotometer (ND-1000, Nanodrop Technologies) and RNA Integrity was determined by gel electrophoresis.

Table 2. The primer sequence of microRNAs

Gene	Primer sequence	Human (5'→3')
miR-4498	RT stem-loop	5-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCAGCAC-3
	Forward	5-GGGTGGGCTGGCAGGGCAA-3
	Reverse	5-CAGTGCGTGTCGTGGAGT-3
miR-3065-3p	RT stem-loop	5-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCTCCAA-3
	Forward	5-GGGTCAGCACCAGGATATTG-3
	Reverse	5-CAGTGCGTGTCGTGGAGT-3
miR-370-5p	RT stem-loop	5-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGTAACT-3
	Forward	5-GGGCAGGTCACGTCTCTGC-3
	Reverse	5-CAGTGCGTGTCGTGGAGT-3
miR-148a-5p	RT stem-loop	5-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGTCGG-3
	Forward primer	5-GGG AAAGTTCTGAGACACT-3
	Reverse	5-CAGTGCGTGTCGTGGAGT-3
miR-222-5p	RT stem-loop	5-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGGATC-3
	Forward	5-GGGCTCAGTAGCCAGTGTA-3
	Reverse	5-CAGTGCGTGTCGTGGAGT-3
miR-223-5p	RT stem-loop	5-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAACTCA-3
	Forward	5-GGGCGTGTATTTGACAAGC-3
	Reverse	5-CAGTGCGTGTCGTGGAGT-3

miRNA labeling and array hybridization

RNA labeling and array hybridization was according to Exiqon's manual. After quality control, the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's guideline for miRNA labelling by following steps: a) 1 μL RNA in 2.0 μL of water was combined with 1.0 μL of CIP buffer and CIP (Exiqon). The mixture was incubated for 30 min at 37°C. b) The Reaction was terminated by incubation for 5 min at 95°C. Then 3.0 μL of labeling buffer, 1.5 μL of fluorescent label (Hy3TM), 2.0 μL of DMSO, 2.0 μL of labeling enzyme were added into the mixture. The labeling reaction was incubated for 1 h at 16°C. c) Terminated by incubation for 15 min at 65°C. After stopping the labeling procedure, the Hy3™-labeled samples were hybridized on the miRCURYTM LNA Array (v.19.0) (Exiqon) according to array manual. a) The total 25 μL mixture from Hy3™-labeled samples with 25 μL hybridization buffer were first denatured for 2 min at 95°C, incubated on ice for 2 min. b) Then hybridized to the microarray for 16-20 h at 56°C in a 12-Bay Hybridization Systems (Hybridization System-Nimblegen Systems, Inc., Madison, WI, USA). c) Following hybridization, the slides were achieved, washed several times using Wash buffer kit (Exiqon). Then the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA).

Quantitative real-time PCR to detect mRNA expression

The PCR procedure followed the previously published steps [14]. Total RNA in MVs was isolated by using RNAiso Plus according to the manufacturer's protocol. The cDNAs were produced with a PrimeScript RT reagent kit and incubated at 37°C for 15 min and 85°C for 5 s. Real-time PCRs were performed using a StepOne Plus device (Applied Biosystems) at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s, according to the instructions for the SYBR Premix Ex Taq kit. The data were analyzed by the $2^{-\Delta\Delta CT}$ method [15]. All the primers were synthesized by GenScript (Nanjing, China), and the sequences are shown in **Table 2**.

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses of possible 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. Enrichment provided a measure of the significance of the function. The Gene Ontology proj-

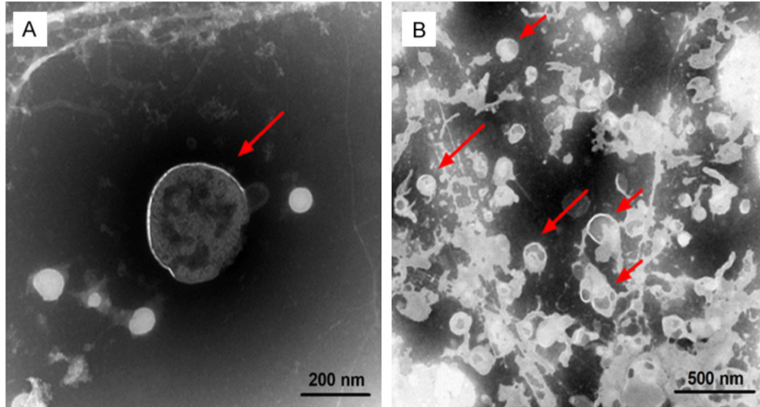

Figure 1. The diameters of MVs from circulation were 100~1000 nm.

ect provides a controlled vocabulary to describe gene and gene product attributes in any organism (http://www.geneontology.org). The ontology covers three domains: Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). Fisher's exact test is used to find if there is more overlap between the DE list and the GO annotation list than would be expected by chance. The *p*-value denotes the significance of GO terms enrichment in the DE genes. The lower the *p*-value, the more significant the GO Term (*p*-value ≤ 0.05 is recommended). Pathway analysis is a functional analysis mapping genes to KEGG pathways. The *p*-value) denotes the significance of the Pathway correlated to the conditions. Lower the *p*-value, more significant is the Pathway. (The recommend *p*-value cut-off is 0.05.)

miRNA target gene prediction and functional analysis

Potential target genes of the differentially expressed miRNAs were predicted from data in the Target Scan 7.1 (http://www.targetscan.org) and mirdbV5 (http://www.mirdb.org/) databases, and the final targets were integrated from these two public databases.

MiRNA gene regulatory network between different miRNA molecules and target genes

Based on the Targetscan and mirdb 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

Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNA molecules were averaged and miRNAs with intensities ≥ 30 in all samples were chosen for calculation normalization factor. 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 2 times higher or 0.5 times lower, respectively, than that of the control group ($P < 0.05$).

The data are expressed as mean ± standard deviation (SD). The t-test was used for comparison between two groups. Data analysis was performed using SPSS13.0 statistical software (SPSS, Inc.). Statistical significance was set at $P < 0.05$ [16].

Results

Morphology of circulating mvs and quality detection of RNA within MVs

We isolated MVs from blood samples using specific centrifugal method as previous reported. Then in this study, the MVs isolated from the peripheral blood of HBV patients and normal people were termed as circulation-derived MVs. The presence of MVs was identified by electron microscopy. The diameters of MVs from circulation of HBV and normal control were both 100~1000 nm (**Figure 1**), which is consistent with the report [17].

Expression of miRNAs in MVs of CHB patients

MiRNA microarray was used to detect miRNA expression in MVs of CHB patients. Compared with the controls, 101 significantly up-regulated and 120 significantly down-regulated miRNAs were identified in MVs of CHB patients (fold change ≥ 2 and $P < 0.05$). And we respec-

Table 3. The 30 significantly dysregulated miRNAs in the MVs of CHB patients

ID		Fold change	Regulation	*P*-Value
168854	hsa-miR-4498	78.14714901	Up	4.15396E-06
147903	hsa-miR-3065-3p	44.38868791	Up	0.018229387
148300	hsa-miR-370-5p	35.33785696	Up	0.010422493
31076	hsa-miR-559	26.19089771	Up	0.000659722
145844	hsa-miR-374a-5p	23.81163294	Up	0.001609915
147262	hsa-miR-548h-3p/hsa-miR-548z	20.71617262	Up	0.012080361
11144	hsa-miR-512-3p	19.75156102	Up	0.043269942
29529	hsa-miR-369-3p	17.35836679	Up	0.004653786
42669	hsa-miR-505-3p	16.32176388	Up	0.019030316
17358	ebv-miR-BART16	16.19164207	Up	0.028889391
46415	hsa-miR-548l	15.39998996	Up	0.001369958
168716	hsa-miR-4487	15.00070768	Up	0.025982142
168831	hsa-miR-433-3p	14.19921702	Up	0.013201882
42556	hsa-miR-889-3p	13.73812286	Up	0.014808222
42756	hsa-miR-548d-5p	12.6018372	Up	0.008753093
17872	hsa-miR-148a-5p	0.009567159	Down	0.005202562
17918	hsa-miR-222-5p	0.017014533	Down	7.9898E-05
42460	hsa-miR-223-5p	0.020354353	Down	0.004378148
13171	hsa-miR-429	0.029341801	Down	0.001806261
147886	hsa-miR-3122	0.035178918	Down	0.011967704
147862	hsa-miR-3158-3p	0.042141838	Down	0.019416848
17888	hsa-let-7a-3p	0.044953973	Down	0.005740744
148684	hsa-miR-628-3p	0.050277506	Down	0.001694302
145972	hsa-miR-141-5p	0.057223494	Down	0.015122881
42874	hsa-miR-16-2-3p	0.059027683	Down	6.5394E-06

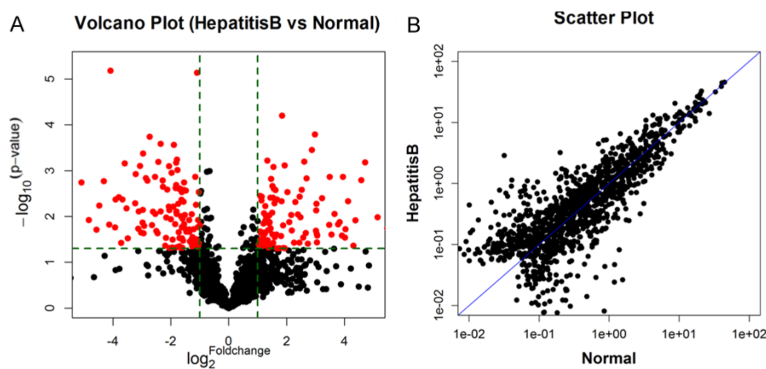

Figure 2. Volcano plot and scatter plot of the miRNA microarray analysis. A. Volcano plots are a useful tool for visualizing differential expression patterns between two conditions. The vertical lines correspond to 1.5-fold up- and down-regulation, and the horizontal line represents a *p*-value of 0.05. Thus, the red point in the plot represents the differentially expressed miRNAs that reached significance. B. The scatter plot is a useful visualization for assessing the variation (or reproducibility) between chips. The axes of the scatter plot are the normalized signal values of the samples (the ratio scale).

tively selected the biggest 15 fold change up-regulated and down-regulated miRNAs to make hierarchical cluster analysis was performed to analyze the resulting data (**Figure 3**). The 30 significantly dysregulated miRNAs in the MVs of CHB patients in the normalized primary microarray data are shown in **Table 3.**

A scatter plot was generated to assess the quality of the miRNA data after filtering, and a volcano plot was generated to visualize the differential expression between two different conditions (**Figure 2**).

Quantitative real-time PCR results

The dysregulated miRNAs (miR-4498, miR-3065-3p, miR-370-5p, miR-148a-5p, miR-222-

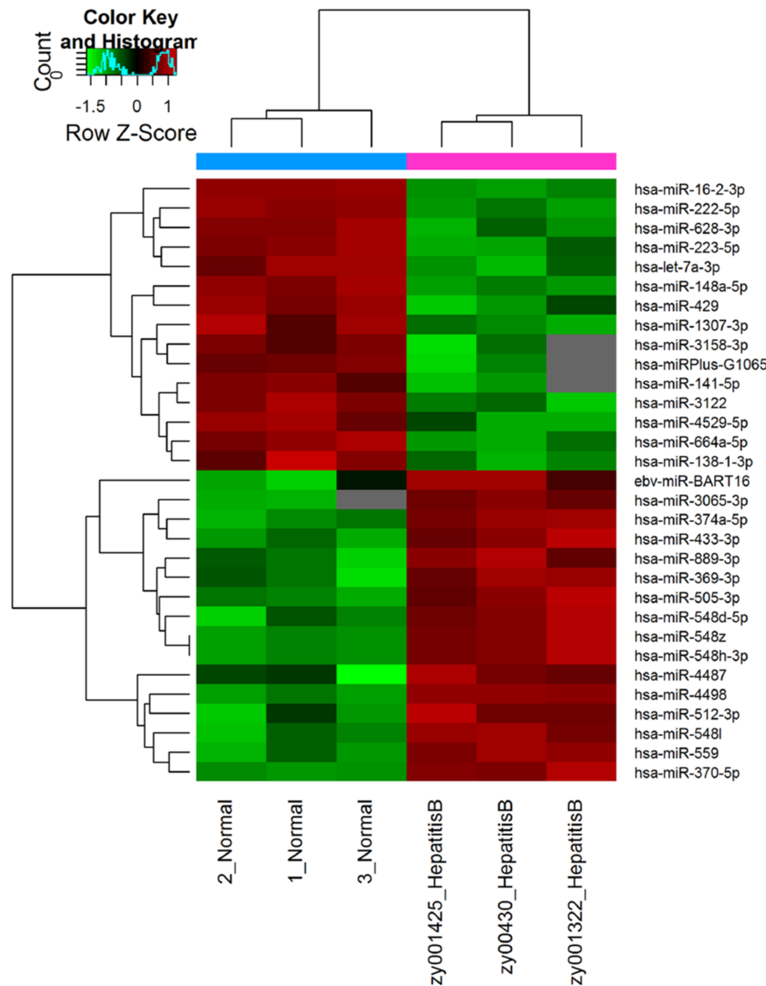

Figure 3. Cluster analysis. Hierarchical cluster analysis using total miRNA of PBMC in CHB and HC. CHB, chronic hepatitis B. NC, normal control. Note: 1. Red represents that the upregulation of miRNA; Green represents a downregulation of miRNA. 2. The PBMCs of three subjects from the same group were pooled and four or three pools were analyzed.

5p and miR-223-5p) were confirmed using RT-PCR and the results were consistent with the microarray analysis. According to the PCR results, compared with the MVs from normal people, miR-4498, miR-3065-3p and miR-370-5p were obviously increased (P < 0.01), and miR-148a-5p, miR-222-5p and miR-223-5p were obviously decreased (P < 0.01) in the MVs from CHB patients. From our results, the miR-4498 and miR-148a-5p presented the most pronounced changes in expression in the hippocampus, which is consistent with the microarray findings (**Figure 4**).

GO or pathway analysis of target genes of regulated miRNAs

The molecular functions of regulated miRNA target genes were analyzed by GO online. The results show that the molecular functions of target genes of regulated miRNAs for BP were mainly related with negative regulation of transcription from RNA polymerase II promoter, peripheral nervous system neuron development, peripheral nervous system neuron differentiation, regulation of mesenchymal cell apoptotic process, negative regulation of fibroblast growth factor receptor signaling pathway, cerebellar Purkinje cell differentiation, cerebellar Purkinje cell layer formation, cerebellar granular layer development, mechanosensory behavior, and Nephron tubule formation. CC were mainly related with PRC1 complex, exocyst, COPI-coated vesicle, SNARE complex, cytoplasmic stress granule, phosphatase complex, protein serine/threonine phosphatase complex, cell surface furrow, cleavage furrow, cell division site part and cell division site. MF were mainly related with vascular endothelial growth factor receptor binding, activin binding, sphingolipid binding, protein phosphatase type 1 regulator activity, eukaryotic initiation factor 4E binding, neuropeptide Y receptor activity, MAP kinase phosphatase activity, tau protein binding, protein serine/threonine phosphatase inhibitor activity and neurotransmitter: sodium symporter activity (**Figure 5**).

Pathway analysis of KEGG showed that the molecular pathways regulating mainly included the Transcriptional misregulation in cancer, Pathways in cancer, Renal cell carcinoma, MAPK signaling pathway, FoxO signaling pathway, TNF signaling pathway, Ras signaling pathway, AMPK signaling pathway, Pancreatic cancer, Herpes simplex infection (**Figure 5**).

Prediction of target genes of miRNAs

The intersections of miRNA target genes were analyzed by TargetScan7.1 and mirdbV5. The target genes in TargetScan7.1 database was

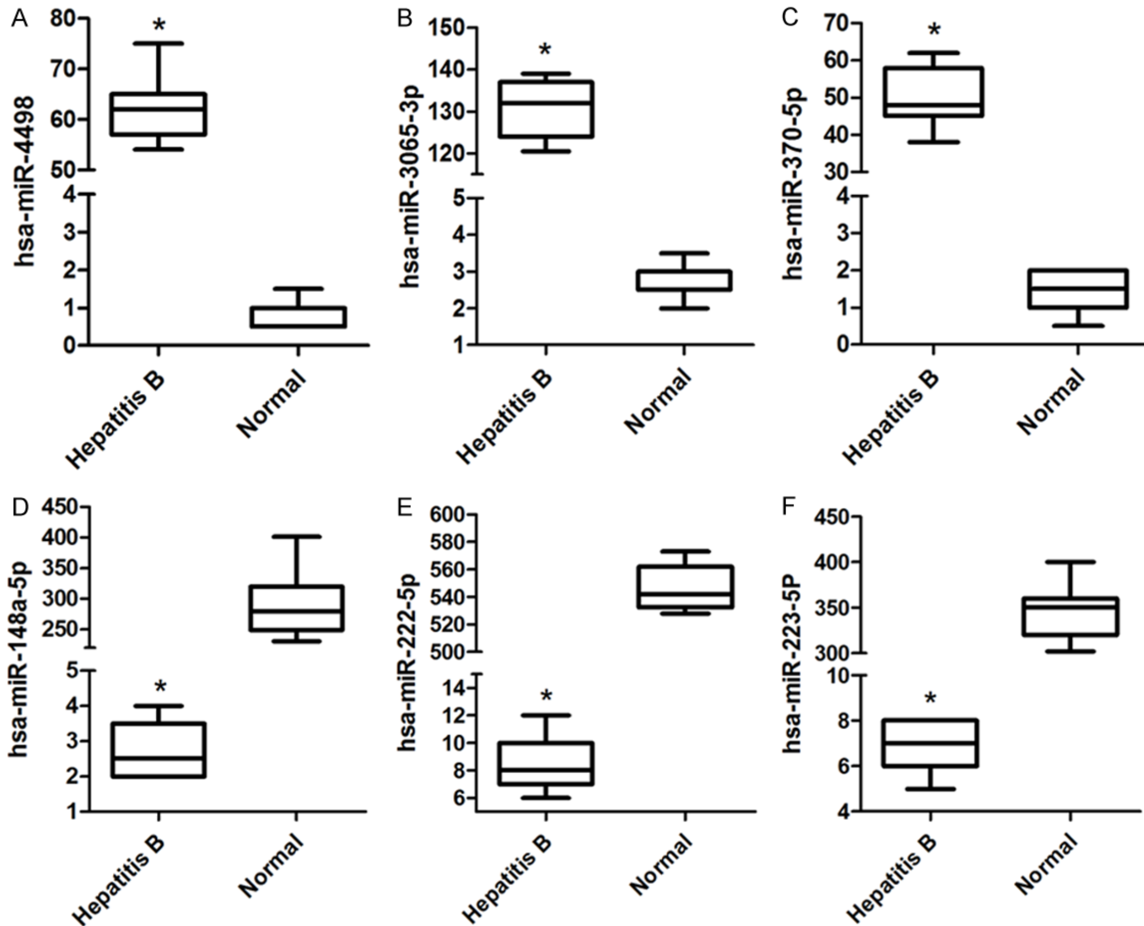

Figure 4. The dysregulated miRNAs (miR-4498, miR-3065-3p, miR-370-5p, miR-148a-5p, miR-222-5p and miR-223-5p) were confirmed using RT-PCR. According to the PCR results, compared with the MVs from normal people, miR-4498, miR-3065-3p and miR-370-5p were obviously increased, and miR-148a-5p, miR-222-5p and miR-223-5p were obviously decreased in the MVs from normal people. The miR-4498 and miR-148a-5p presented the most pronounced changes in expression in the hippocampus, which is consistent with the microarray findings. Data were expressed as the mean ± SD. n = 30. (*$P < 0.01$ compared to the to the Normal group).

4900 and the target genes in mirdbV5 database was 5724. The number of common target genes was 1289 (**Figure 6**).

Analysis of miRNA-mRNA network of regulated miRNA molecules

The miRNA-gene-network was constructed based on the relationship between regulated miRNAs and their target genes. The results show that the up-regulated miRNA molecules (has-miR-4498, has-miR-3065-3p, miR-370-5p etc.) and down-regulated miRNA molecules (has-miR-148a-5p, has-miR-222-5p, has-miR-223-5p, 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-1RAP IL-2, IL-6, IL-6R, IL-8, IL-13, IL-22, IFN-2, TNF-AIP1 etc., but also important gene associated with immune signaling pathways, such as STAT-3, SOCS etc., molecules in JAK-STAT signaling pathway associated with interferon and cytokine; TNFRSF, TNFRSF19, TRAM, Bcl-6, etc. in NF-κB signal pathway, TLR signal pathway and Trim-2, Trim-5, Trim-6 molecules of TRIM protein family (**Figure 7**).

Discussion

The enhancement of immune cells, mainly specific cytotoxic T cells, Th1, and Treg cell, are related to immune tolerance to immune clearance in the procession of chronic HBV infection [18]. MiRNA-146a, miRNA-150 and miRNA-155 miRNA molecules derived from immune cells play an important role in the differentiation

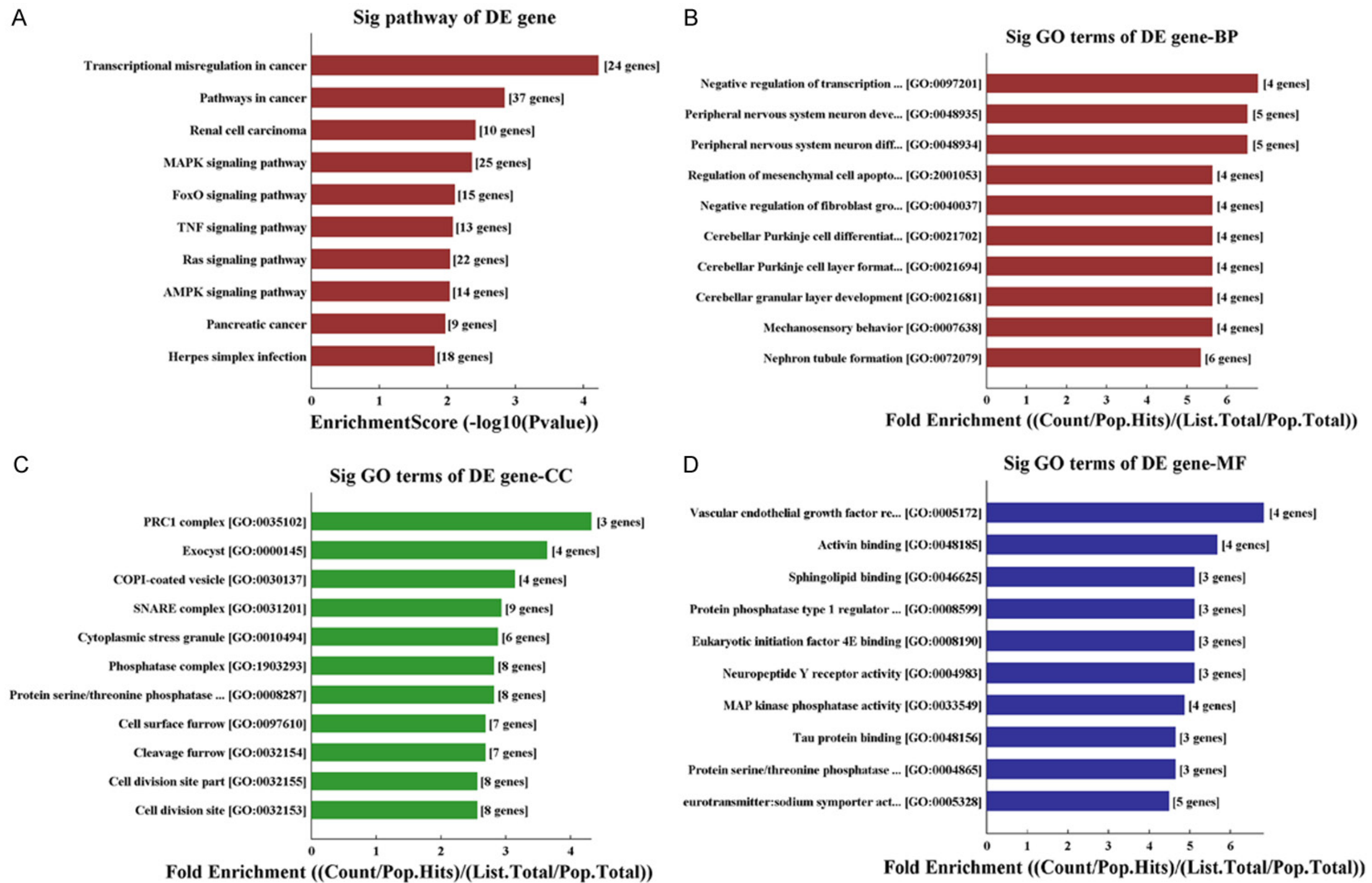

Figure 5. GO and KEGG analyses of possible regulated target genes. A. Signal pathway of DE gene. B. Signal Go terms of DE gene-Biological Process (BP). C. Signal Go terms of DE gene-Cellular Component (CC). D. Signal Go terms of DE gene-Molecular Function (MF).

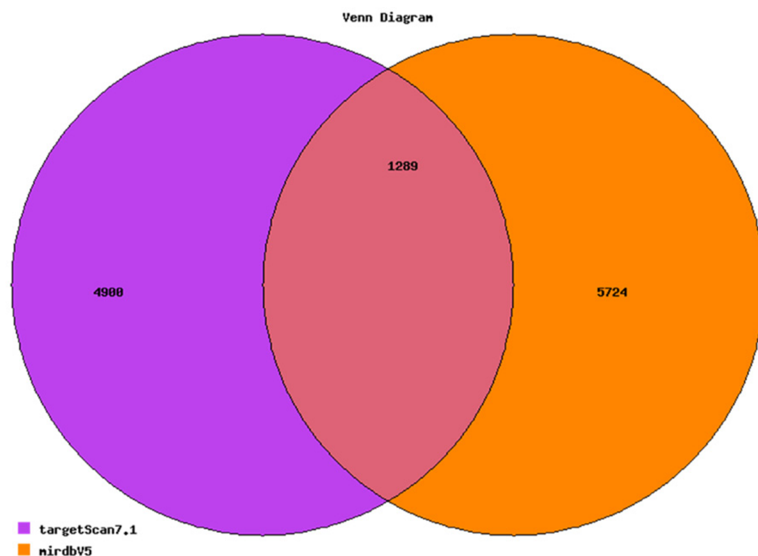

Figure 6. The intersections of miRNA target genes were analyzed by TargetScan7.1 and mirdbV5. The target genes in TargetScan7.1 was 4900 and the target genes in mirdbV5 was 5724. The number of common target genes was 1289.

and maturation of immune cells [19, 20]. MiRNA-122, miRNA-192, and miRNA-455 derived from circular peripheral blood were enriched in the plasma of patients with HBeAg-positive CHB in children [21]. Some studies have showed that the expression of hsa-miR-548ah-5p and hsa-miR-29b-3p are related to the pathogenesis of CHB [22, 23]. Hsa-miR-548ah-5p and hsa-miR-29b-3p regulate immune function by regulating the expression of IFN-λ1 and IFN-g, respectively.

But the function of microRNA derived from MVs is rarely reported. The function of MVs is dependent on the cargo they carry, such as microRNA, and the diagnostic and prognostic roles of peripheral circulating MVs for CHB remain unclear. So we detected the miRNA expression of MVs in CHB patients and analysis the MVs drived miRNAs and its target genes in immune clearance in the procession of chronic HBV patients.

In our study, we use MiRNA microarray to detect miRNA expression in MVs of CHB patients, and find 101 significantly up-regulated (i.e., miR-4498, miR-3065-3p, and miR-370-5p) and 120 significantly down-regulated miRNAs (i.e., miR-148a-5p, miR-222-5p and miR-223-5p) were identified in MVs of CHB patients (fold change ≥ 2 and P < 0.05). Moreover, in order to verify whether the microRNAs are consistent with the microarray analysis express differently in 15 CHB patients compared with 15 normal people, RT-PCR technique was used to detect dysregulated miRNAs. According to the PCR results, compared with the MVs from normal people, miR-4498, miR-3065-3p and miR-370-5p were obviously increased (P < 0.01), and miR-148a-5p, miR-222-5p and miR-223-5p were obviously decreased (P < 0.01) in the MVs from normal people, which suggests these MVs-drived microRNA molecules may be used as important index of diagnosing CHB.

Functional enrichment analysis showed that the molecular functions of target genes by regulated miRNA molecules for BP were mainly related with negative regulation of transcription from RNA polymerase II promoter, peripheral nervous system neuron development, peripheral nervous system neuron differentiation, regulation of mesenchymal cell apoptotic process, negative regulation of fibroblast growth factor receptor signaling pathway, cerebellar Purkinje cell differentiation, cerebellar Purkinje cell layer formation, cerebellar granular layer development, mechanosensory behavior, and Nephron tubule formation. CC were mainly related with PRC1 complex, exocyst, COPI-coated vesicle, SNARE complex, cytoplasmic stress granule, phosphatase complex, protein serine/threonine phosphatase complex, cell surface furrow, cleavage furrow, cell division site part and cell division site. MF were mainly related with vascular endothelial growth factor receptor binding, activin binding, sphingolipid binding, protein phosphatase type 1 regulator activity, eukaryotic initiation factor 4E binding, neuropeptide Y receptor activity, MAP kinase phosphatase activity, tau protein binding, protein serine/threonine phosphatase inhibitor activity and neurotransmitter: sodium symporter activity. Further analysis of the signal transduction pathway of target genes revealed that the molecular pathways regulated by miRNA-regulated molecules mainly included the Transcriptional misregulation in cancer, Pathways in cancer, Renal cell carcinoma, MAPK signaling pathway, FoxO signaling path-

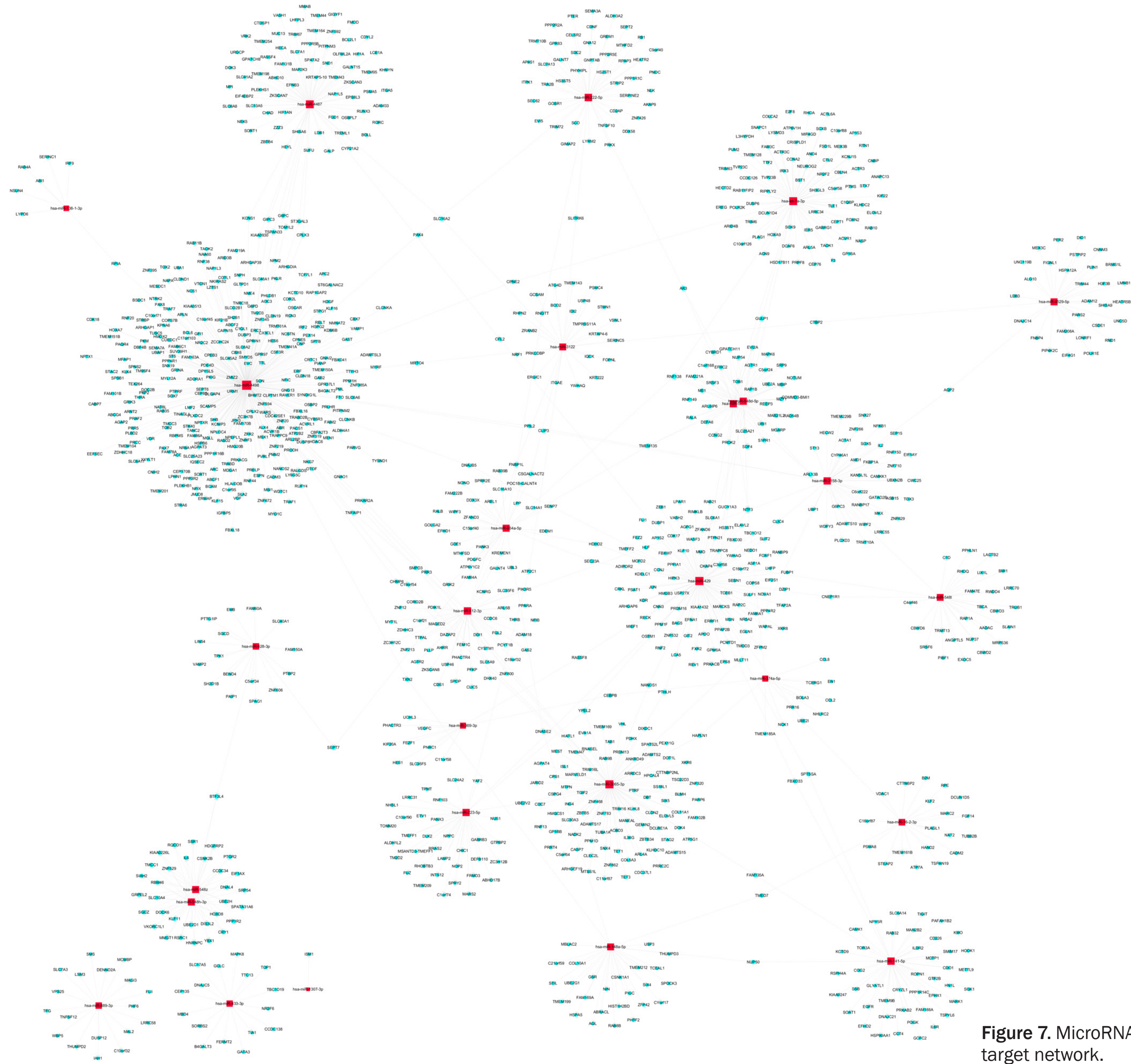

Figure 7. MicroRNA target network.

way, TNF signaling pathway, Ras signaling pathway, AMPK signaling pathway, Pancreatic cancer, Herpes simplex infection. And those 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 the regulatory effects of miRNA molecules are pleiotropic and miRNA molecules mainly play their regulatory functions at post-transcription. The related cytokines played important roles in immune function, such as IL-1RAP IL-2, IL-6, IL-6R, IL-8, IL-13, IL-22, IFN-2, TNF-AIP1 etc. And the important gene associated with immune signaling pathways, such as STAT-3, SOCS etc., molecules in JAK-STAT signaling pathway associated with interferon and cytokine; TNFRSF [24], TNFRSF19, TRAM, Bcl-6, etc. in NF-κB signal pathway, TLR signal pathway and Trim-2, Trim-5, Trim-6 molecules of TRIM protein family. The aforementioned results suggested that miRNA expression in MVs 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 expressions of miRNA molecules in MVs 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.

Address correspondence to: Dr. Xiao-Dong Li, Department of Hepatology, Hubei Provincial Hospital of Chinese Medicine, No. 4 Huayuanshan, Wuchang district, Wuhan 430061, PR China. Tel: +86 13908658127; E-mail: lixiaodong555@126.com

References

[1] Grimm D, Thimme R, Blum HE. HBV life cycle and novel drug targets. Hepatol Int 2011; 5: 644-53.

[2] Kang L, Pan J, Wu J, Hu J, Sun Q, Tang J. Anti-HBV drugs: progress, unmet needs, and new hope. Viruses 2015; 7: 4960-77.

[3] WHO. Guidelines for the prevention, Care and treatment of persons with chronic hepatitis B infection. Geneva 2015.

[4] Lu FM, Zhuang H. Management of hepatitis B in China. Chinese Medical Journal 2009; 122: 3-4.

[5] Fiorino S, Bacchi-Reggiani ML, Visani M, Acquaviva G, Fornelli A, Masetti M, Tura A, Grizzi F, Zanello M, Mastrangelo L, Lombardi R, Di Tommaso L, Bondi A, Sabbatani S, Domanico A, Fabbri C, Leandri P, Pession A, Jovine E, de Biase D. MicroRNAs as possible biomarkers for diagnosis and prognosis of hepatitis B- and C-related-hepatocellular-carcinoma. World J Gastroenterol 2016; 22: 3907-36.

[6] Xie KL, Zhang YG, Liu J, Zeng Y, Wu H. MicroRNAs associated with HBV infection and HBV-related HCC. Theranostics 2014; 4: 1176-92.

[7] Burger D, Schock S, Thompson CS, Montezano AC, Hakim AM, Touyz RM. Microparticles: biomarkers and beyond. Clin Sci (Lond) 2013; 124: 423-41.

[8] Lemoinne S, Thabut D, Housset C, Moreau R, Valla D, Boulanger CM, Rautou PE. The emerging roles of microvesicles in liver diseases. Nat Rev Gastroenterol Hepatol 2014; 11: 350-61.

[9] Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C. Microvesicles: mediators of extracellular communication during cancer progression. J Cell Sci 2010; 123: 1603-11.

[10] Chinese liver disease association and Chinese medical association infectious disease branch: chronic hepatitis B prevention guide (2015 Edition). Chinese Journal of Hepatology 2015; 7: 1-15.

[11] Xing TJ, Wang B, Zhang J. Screening and bioinformatics analysis of microRNA-associated immune clearance in patients with chronic hepatitis B. Int J Clin Exp Med 2014; 7: 4924-4932.

[12] Sarin SK, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, Jafri W, Jia J, Kim JH, Lai CL, Lee HC, Lim SG, Liu CJ, Locarnini S, Al Mahtab M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma BC, Sollano J, Wang FS, Wei L, Yuen MF, Zheng SS, Kao JH. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. Hepatol Int 2016; 10: 1-98.

[13] Lu L, Guo D, Chen X, Xiong W, Jie S, Li H. Abnormal miRNAs targeting chromosome open reading frame genes were enriched in microvesicles derived from the circulation of HCC. Biochem Genet 2016; 54: 120-33.

[14] Wang Y, Yang F, Xue J, Zhou X, Luo L, Ma Q, Chen YF, Zhang J, Zhang SL, Zhao L. Anti-Schistosomiasis liver fibrosis effects of chlorogenic acid through IL-13/miR-21/Smad7 signaling interactions in vivo and in vitro. Antimicrob Agents Chemother 2017; 61: AAC.01347-16.

[15] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C (T) method. Nat Protoc 2008; 3: 1101-1108.

[16] Yang F, Wang Y, Xue J, Ma Q, Zhang J, Chen YF, Shang ZZ, Li QQ, Zhang SL, Zhao L. Effect of Corilagin on miR-21/smad7/ERK signal pathway in schistosomiasis-induced hepatic fibrosis in mouse model. Parasitol Int 2016; 65: 308-315.
[17] Han CZ, Juncadella IJ, Kinchen JM, Buckley MW, Klibanov AL, Dryden K, Onengut-Gumuscu S, Erdbrügger U, Turner SD, Shim YM, Tung KS, Ravichandran KS. Macrophages redirect phagocytosis by non-professional phagocytes and influence inflammation. Nature 2016; 539: 570-574.
[18] Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology 2007; 45: 507-539.
[19] Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, Yoshimura A, Baltimore D, Rudensky AY. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. Cell 2010; 142: 914-929.
[20] Dai R, Ahmed SA. microRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. Transl Res 2011; 157: 163-179.
[21] Winther TN, Bang-Berthelsen CH, Heiberg IL, Pociot F, Hogh B. Differential plasma microRNA profiles in HBeAg positive and HBeAg negative children with chronic hepatitis B. PLoS One 2013; 8: e58236.
[22] Li Y, Xie J, Xu X, Wang J, Ao F, Wan Y, Zhu Y. MicroRNA-548 down-regulates host antiviral response via direct targeting of IFN-λ1. Protein Cell 2013; 4: 130-141.
[23] Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, Hua M, Li N, Yao H, Cao X. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-γ. Nat Immunol 2011; 12: 861-869.
[24] Guo P. Suppression of interferon-mediated antiviral immunity by hepatitis B virus: an overview of research progress. Scand J Immunol 2013; 78: 230-23.