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Original Article

Screening and bioinformatic analysis of differentially expressed circRNA molecules in different stages of chronic hepatitis B

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Abstract: *Objective:* This study aimed to screen differential expressions of circular RNA (circRNA) at different stages of chronic hepatitis B (CHB) and explore the possible roles of circRNAs in regulating the immune responses in CHB patients. *Methods:* Expressions of circRNAs were detected in peripheral blood mononuclear cells (PBMC) at different stages of CHB in patients using a new generation of circRNA microarray. Results from circRNA microarray were validated using quantitative fluorescence polymerase chain reaction. circRNA/microRNA (miRNA) interaction was predicted using Arraystar's homemade miRNA target prediction software. Target genes regulated by circRNA-related miRNA were analyzed by Gene Oncology (GO) and Pathway analyses. *Results:* Compared with healthy controls, 137 differentially expressed circRNAs, of which 89 were upregulated, and 48 were downregulated, were detected in PBMC of CHB patients. A total of 444 differentially expressed circRNAs were detected in PBMC of chronic hepatitis B virus (HBV) carriers. Compared with chronic HBV carriers, 1,041 differentially expressed circRNAs were detected in CHB patients. Many miRNA response elements can be complementarily paired with seed regions on miRNA in different circRNAs. Target gene analysis showed relationship with miRNAs of 533 target genes regulated by hsa_circ_0038646 and 249 target genes regulated by hsa_circ_0087354. GO analysis showed that target genes regulated by miRNA-related hsa_circ_0038646 mainly enriched activin binding, whereas target genes regulated by miRNA-related hsa_circ_0087354 mainly enriched armadillo repeat domain binding. Pathway analysis showed that miRNA-related hsa_circ_0038646 molecules was mainly involved with T cell receptor signaling pathway and estrogen receptor signaling pathway. miRNA-related hsa_circ_0087354 was mainly involved with adherens junction and mitogen-activated protein kinase signaling pathway. There were positive correlation between the expression of hsa_circ_0038646 and ALT. *Conclusion:* Differentially expressed circRNAs in PBMC were detected at different stages of CHB patients. Differential expression of circRNA may be involved in the immune regulation of chronic HBV infection through regulation of multiple target genes and signaling pathways.

Keywords: Chronic hepatitis B, circular RNA, immune regulation

Introduction

Chronic hepatitis B (CHB) is one of the most significant infectious diseases caused by hepatitis B virus (HBV) infection. According to the latest data released by the World Health Organization (WHO), approximately 240 million people are infected with chronic HBV, and 20% to 30% of these cases may progress to cirrhosis and/or liver cancer. The WHO estimated that 650,000 people die annually because of cirrhosis and liver cancer caused by CHB [1]. In recent years, anti-HBV treatment made considerable progress, particularly in application of potent low-resistance drugs and significant improvement in prognosis of CHB patients. However, some problems, such as the need for long-term application and recurrence after discontinuation, must still be addressed [2]. HBV is a kind of noncytotoxic virus. Liver injury may result from immune response of the body, particularly HBV-specific T cell reaction that determines outcome of HBV infection, but its specific mechanism remains incompletely elucidated [3]. Therefore, further studies should resolve pathogenesis of CHB and development of new molecular therapies.

Circular RNAs (circRNAs) are recently discovered non-encoding molecular RNAs that widely

Table 1. Baseline data of three groups

Group	n	Sex (m/f)	Age (y)	ALT (U/L)	HBV-DNA (lgIU/mL)
CHB	4	4/0	39±5	519±511	5.6±1.6
HBVC	3	2/1	33±6	23.7±11	6.8±1.7
HC	3	0/3	36±6	21.2±7.8	-
F/x^2	-	0.40	1.02	2.64	1.13
P	-	0.754	0.367	0.14	0.309

CHB: Chronic hepatitis B; HBVC: Hepatitis B virus carrier; HC: Healthy control.

Table 2. The primer sequences of RT-PCR

Gene	Primer sequence	Length (bp)
β-actin (Human)	F: 5'GTGGCCGAGGACTTTGATTG3'	73
	R: 5'CCTGTAACAACGCATCTCATATT3'	
hsa_circ_0087354	F: 5'CTGGAGTAGGAGTTTGGTGGTA3'	64
	R: 5'CTTCACCAGAGGATGTATTGCT3'	
hsa_circ_0038646	F: 5'TATGGTGGAGAAGCGGGTGTT3'	137
	R: 5'CCAGGGCAGGAGAATGTGA3'	

exist in eukaryotic cells. circRNAs are highly conserved, stable, and tissue specific and originate from exons and introns. circRNAs from exons contain rich binding sites for microRNAs (miRNAs), act as miRNA sponge, and regulate miRNA function [4, 5]. miRNAs regulate mRNA expression at the posttranscriptional level and control differentiation of immune cells. miRNAs also participate in regulation of immune response [6, 7]. miRNAs play important roles in tumor pathogenesis, infectious diseases, and other conditions [8]. However, no study reported the role of circRNAs in immune regulation response in chronic HBV infection.

Several studies showed that miRNAs play key roles in chronic HBV infection and liver injury [9, 10]. circRNAs are speculated to participate in pathogenesis of CHB as direct regulators of miRNA molecules. Stability of circRNAs indicates their potential as molecular markers. Therefore, circRNA expressions were screened in peripheral blood mononuclear cells (PBMC) at different stages in CHB patients using circRNA expression chips. Functions of genes and signaling pathways were analyzed using a biological information software, and possible role of circRNA molecules in regulation of immune response of CHB was investigated. Potentially new molecular targets may be obtained for treatment of CHB.

Subjects and methods

Subjects

Blood samples were collected from four CHB patients and three CHB virus carriers at the Taizhou People's Hospital from October 2014 to October 2015. Healthy controls consisted of three cases. **Table 1** showed the baseline data of the three groups. Written informed consent was obtained from all subjects. Experimental protocol was approved by the ethical commission of Taizhou People's Hospital. Diagnostic criteria were based on the 2010 Chronic Hepatitis B Prevention Guide of China [11]. All patients were negative for antibodies against hepatitis A, C, D and E viruses and human immunodeficiency virus. Excluded patients comprised all individuals with history and clinical features of drug-induced liver injury, alcoholic hepatitis, liver tumor, and steatohepatitis and those treated with nucleot(s)ide analogs and antiviral or immunomodulatory drugs in the previous six months.

Isolation of PBMC

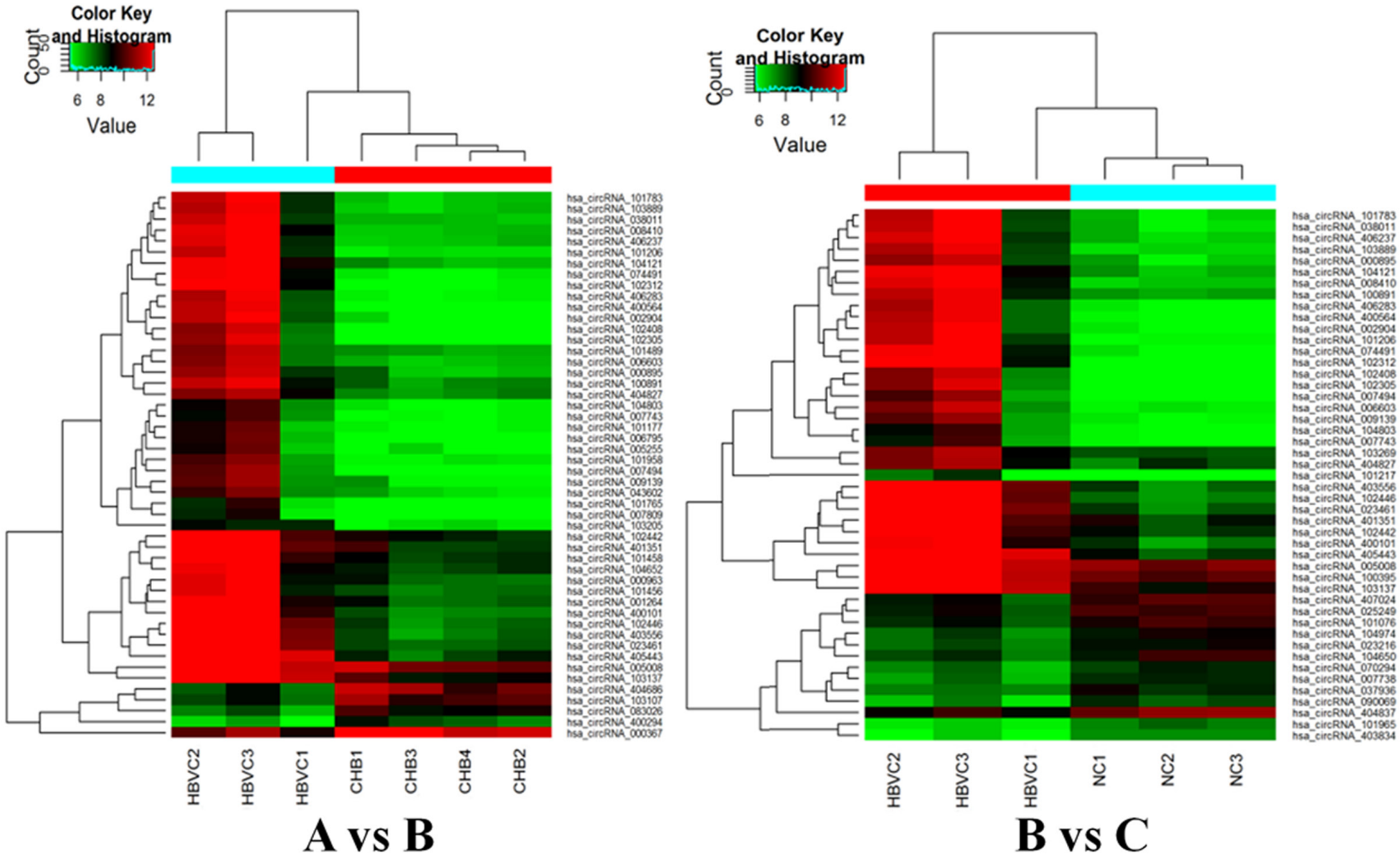
PBMC was isolated from the peripheral blood of all subjects by density gradient centrifugation.

Extraction and quantification of RNA in PBMC

Total RNA in PBMC was extracted using TRIzol reagent (Invitrogen Life Technologies, Waltham, MA, USA). Concentrations of RNA samples were determined by OD260 using NanoDrop ND-1000 instrument. Integrity of RNA was assessed by electrophoresis on a denaturing agarose gel.

Labeling and hybridization of circRNA chip

Sample labeling and array hybridization were performed according to manufacturer's protocol (Arraystar Inc., Rockville, MD, USA). Briefly, total RNAs were digested with RNase R (Epicentre Inc., Madison, WI, USA) to remove linear RNAs and to enrich circRNAs. Then, enriched circRNAs were amplified and transcribed into fluorescent cRNA utilizing a ran-


Color Key and Histogram
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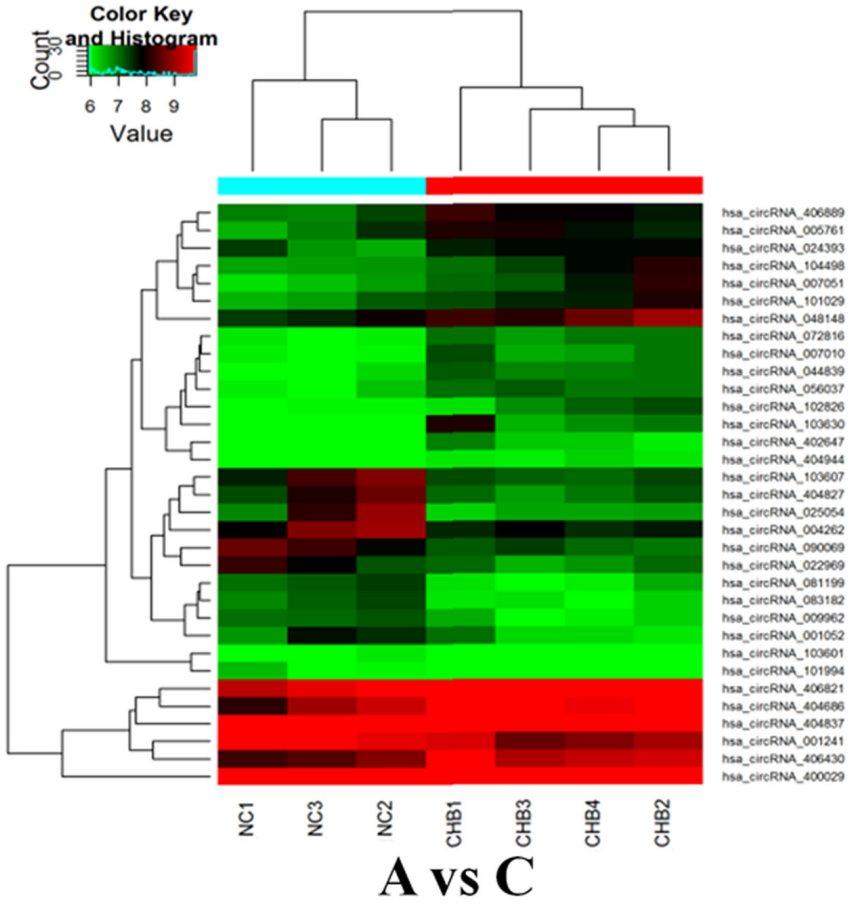

Figure 1. Hierarchical cluster analysis using circRNAs of PBMC in patients with CHB. A. Chronic hepatitits B; B. Hepatitis B virus carrier; C. Healthy controls.

Table 3. Detection of circRNAs in three groups by RT-PCR

Group	n	hsa_circ_0038646	hsa_circ_0087354
CHB	4	0.57±0.19	3.37±0.71
HBVC	3	1.41±0.40	8.21±3.72
HC	3	0.37±0.12	3.62±0.33
F	-	14.22	5.60
P	-	0.003	0.035

CHB: Chronic hepatitis B; HBVC: Hepatitis B virus carrier; HC: Healthy control.

dom priming method (Arraystar Super RNA Labeling Kit; Arraystar Inc., Rockville, MD, USA). Subsequently, 1 μg of each labeled cRNA was fragmented by adding 5 μL of 10 × blocking agent and 1 μL of 25 × fragmentation buffer. The mixture was heated at 60°C for 30 min, and 25 μL of 2 × hybridization buffer was added to dilute the labeled cRNA. Then, 50 μL of hybridization solution was dispensed into gasket slide and assembled on circRNA expression microarray slide. Slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven. Hybridized arrays were washed, fixed, and scanned using Agilent Scanner G2505C. Fold change between groups for each circRNA was computed. circRNAs with 1.5-fold changes and p values < 0.05 were considered significantly differentially expressed.

Detection of circRNAs by real-time quantitative fluorescence polymerase chain reaction (PCR)

All primer sequences were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd., Shanghai, China. **Table 2** listed the used primer sequences. First, cDNA was synthesized. Second, quantitative fluorescence PCR was performed using PCR mixture of 5 μL 2 × Master Mix, 10 μmol/L PCR specific primers of sense and antisense chains, and 0.5 μL water to achieve a total volume of 8 μL. The reaction mixture was placed in a 384-well PCR plates, and circRNA template corresponding to 2 μL cDNA was added and mixed using transient centrifugation. The 384-PCR plate was placed in real-time quantitative fluorescence PCR instrument for PCR. β-Actin was used as internal reference. PCR was performed according to the following procedures: 95°C for 10 min; 40 cycles at 95°C for 10 s and 60°C for 60 s. Concentration of target gene in each sample was divided by concentration of housekeeping gene, i.e., relative content of this gene after correction.

Annotation for circRNA/miRNA interaction

circRNA/miRNA interaction was predicted using Arraystar's homemade miRNA target prediction software based on TargetScan and miRanda. Differentially expressed circRNAs within all comparisons were annotated in detail with the circRNA/miRNA interaction information. Interaction information was located in the folder of Excel file containing differentially expressed circRNAs.

Prediction of target genes related to circRNA-regulated miRNA

TargetScan, miRBase, and miRanda databases were used to predict target genes related to circRNA-regulated miRNAs. Intersection of results of the three databases was obtained.

Gene Oncology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the target genes related to circRNA-regulated miRNA

GO (http://www.geneontology.org) was used to classify molecular function of target genes related to circRNA-regulated miRNAs. Pathway analysis was used to determine significant pathways of differential genes according to KEGG (http://www.genome.jp).

Detection of primary clinical indicators

HBV DNA was detected with quantitative fluorescence PCR kit (Shanghai Kehua Bio-Engineering Limited Company, Shanghai, China). Limit of detection is less than 5×10^2 IU/mL. ABI 7300 Real-Time PCR System (ABI Company, Alameda, CA, USA) was used for quantitative PCR. Serum HBV markers was detected by electrochemiluminescence. Serum biochemical indicators were detected using an automatic biochemical analyzer (Hitachi, Tokyo, Japan).

Statistical analysis

Data were expressed as mean ± standard deviation. The t test was used to compare between two groups, while one-way ANOVA and SNK-q tests were used for multiple comparisons. Pearson correlation was used between variables. Data analysis was performed using

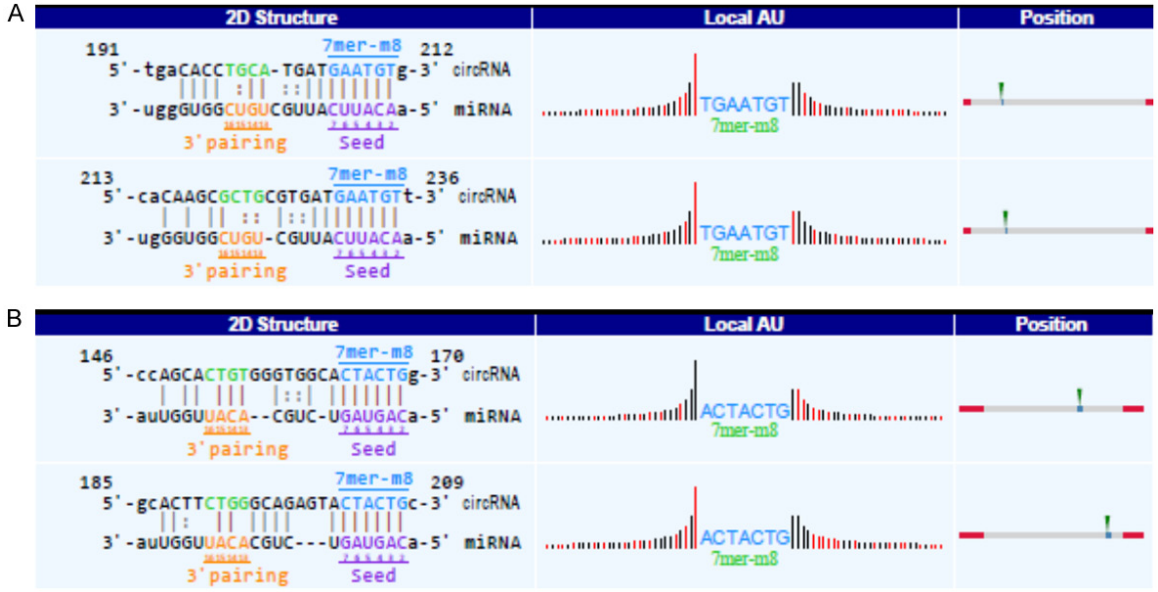

Figure 2. Annotation for hsa_circ_RNA and hsa-miRNA interaction. A. Binding site of hsa_circ_0038646 and hsa-miR-181b-5p; B. Binding site of hsa_circ_0087354 and hsa-miR-199a-3p.

SPSS17.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

Results

Differential expression of circRNAs in PBMC at different stages of CHB

Differential expressions of circRNAs in PBMC of all subjects were screened using the Circular RNA Microarray Version 2 chip. Compared with the healthy control group, 137 differentially expressed circRNAs, of which 89 were upregulated, and 48 were downregulated, were detected in PBMC of CHB patients. A total of 444 differentially expressed circRNAs were detected in chronic HBV carriers; 130 circRNAs were upregulated, including 34 up to more than 5 times, such as hsa_circ_0087354 and hsa_circ_0046968, and 314 were downregulated. Compared with chronic HBV carriers, 1,041 differentially expressed circRNAs were detected in CHB patients. A total of 663 circRNAs were upregulated, and 378 were downregulated, including 54 up to more than 5 times, such as hsa_circ_0038646 and hsa_circ_0041555 (**Figure 1**).

Detection of circRNA expression by quantitative fluorescence PCR

MicroRNAs targeted by all differential circRNAs screened in this study were analysed by bioinformatics. Based on the results of analysis, it was found that the microRNAs targeted by two molecules (hsa_circ_0038646 and hsa_circ_0087354) were miRNA-181 and miRNA-199, respectively. Both of miRNA-181 and miRNA-199 might play an important role in the pathogenesis of immune and liver disease according to current reports. The role of miRNAs targeted by other circ-RNAs in liver disease and immunologic function are indistinct in the present reports. Hence, hsa_circ_0038646 and hsa_circ_0087354 were selected for the investigation. Expression level of hsa_circ_0038646 and hsa_circ_0087354 molecules screened more than five times by the chip were detected using quantitative fluorescence PCR method to verify chip accuracy. Results showed that expression levels of hsa_circ_0038646 and hsa_circ_0087354 in different groups were consistent with the trend of microarray detection, as shown in **Table 3**.

Prediction of target genes related to circRNA regulated miRNA

Arraystar's miRNA prediction software was used to predict the conserved miRNA binding sites on differentially expressed circRNAs. Sites with high scores were annotated. Results showed that the differential expression of circRNAs with miRNA response elements (MRE) could be paired on seed regions of complementary bases with miRNA. circRNA pairs com-

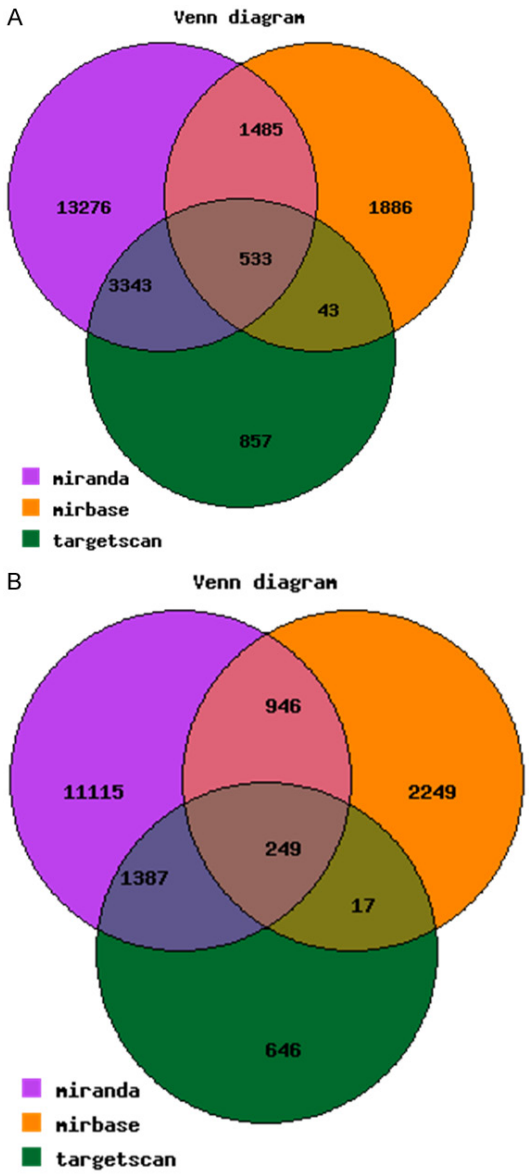

Figure 3. Intersection graphs of target genes regulated by hsa_circ_0038646 and 0087354. A. hsa_circ_0038646; B. hsa_circ_0087354.

plemented a variety of miRNAs, for instance, hsa_circ_0038646 pairs with hsa-miR-181b-5p, hsa-miR-181c-5p, hsa-miR-181d-5p, hsa-miR-181a-5p, and hsa-miR-329-5p (**Figure 2**), whereas hsa_circ_0087354 pairs with hsa-miR-199a-3p, hsa-miR-199b-3p, hsa-miR-449a, hsa-miR-449b-5p, and hsa-miR-630. Various circRNAs paired with miRNAs, such as hsa_circ_0009610 and hsa_circ_0004004, were complementary with hsa-let-7a-2-3p. hsa_circ_003079, hsa_circ_003044, and hsa_circ_0092337 were complementary with hsa-let-7a-3p.

Target genes regulated by hsa_circ_0087354 and hsa_circ_0038646

TargetScan, miRBase, and miRanda databases were used to predict target genes related to the miRNA regulated by hsa_circ_0038646 and hsa_circ_0087354. Results showed the association with miRNA of 533 target genes regulated by hsa_circ_0038646 and 249 target genes regulated by hsa_circ_0087354 (**Figure 3**).

GO analysis of target genes of hsa_circ_0038646 and hsa_circ_0087354

Function analysis of genes related to hsa_circ_0038646- and hsa_circ_0087354-regulated miRNAs was performed using online GO classification tool. Results showed that hsa_circ_0038646 regulating miRNA-related target genes were mainly enriched in activin binding, S100 protein binding, GDP dissociation inhibitor activity, transforming growth factor receptor activity, and ubiquitin protein ligase gene activity, as shown in **Figure 4A**. Results also showed that molecular functions of target genes related to miRNA of hsa_circ_0087354 were enriched in armadillo repeat domain binding, pre-mRNA binding, mitogen-activated protein kinase (MAPK) activity, translation repressor activity, and GTP-dependent protein binding gene activity (**Figure 4B**).

KEGG analysis of target genes of hsa_circ_0038646 and hsa_circ_0087354

Based on KEGG biological pathway enrichment analysis and *p* value calculation using Fisher's exact test, results showed that miRNA-related hsa_circ_0038646 was mainly involved in T cell receptor binding pathway, estrogen receptor signaling, phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling, and axon guidance signals (**Figure 5A**). Results also showed that miRNA-related hsa_circ_0087354 mainly participated in adherens junction, MAPK signaling pathway, central carbon metabolism in cancer, Notch signaling pathway, and gonadotropin-releasing hormone (GnRH) signaling pathway (**Figure 5B**).

Correlation analysis between two kinds of circRNA and ALT or HBVDNA

The level of ALT and HBVDNA in 4 CHB patients was (519±511) U/L, (6.9±0.78) lgIU/ml,

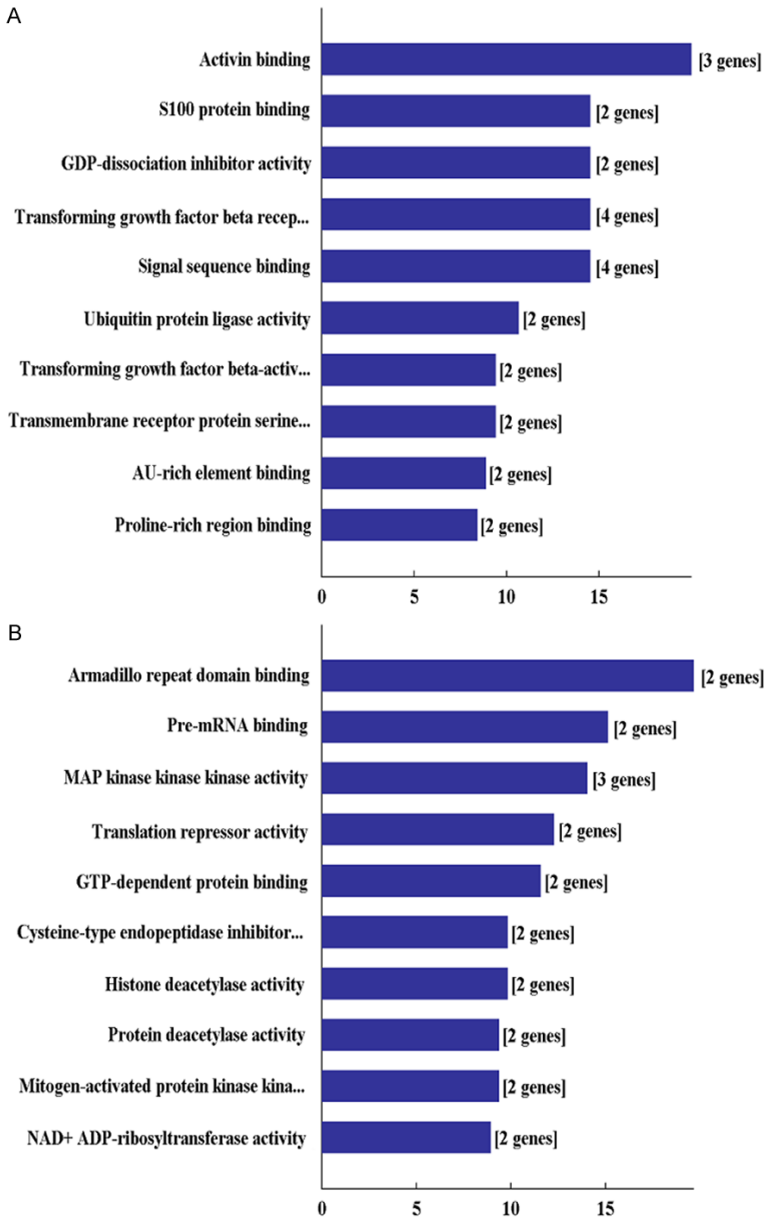

Figure 4. Molecular function of Go analysis of hsa_circ_0038646 and 0087354 target gene. A. hsa_circ_0038646; B. hsa_circ_0087354.

respectively. Positive correlation was found between the expression of hsa_circ_0038646 and ALT (r=0.973, *P*=0.027) (**Figure 6**). No correlation was found between the expression of hsa_circ_0038646 and HBVDNA (r=0.256, *P*=0.744). There were no correlation between the expression of hsa_circ_ 0087354 and ALT or HBV DNA (r=0.45, *P*=0.547 and r=0.707, *P*=0.293, respectively).

Discussion

Natural history of CHB includes immune tolerance, immune clearance, and immune control. Immune functions differ at different stages in CHB patients. However, molecular mechanisms leading to differences remain unclear [12]. circRNAs are involved in the complex RNA-RNA interaction network and play important roles in regulatory function of gene transcription [13]. Several studies showed that circRNAs take part in development and progression of diseases, such as cancer, and the nervous system through their interaction with miRNAs [14-16]. In this study, differential expressions of circRNAs were screened in PBMC at different stages of CHB in studied patients. Results showed that compared with the healthy control group, 137 differentially expressed circRNAs were detected in PBMC of CHB patients, and another 444 differentially expressed circRNAs were detected in chronic HBV carriers. Especially, differential expression of circRNAs more obvious between the chronic HBV carriers and CHB patients. Results of quantitative fluorescence PCR showed that expression levels of hsa_circ_0038646 and hsa_circ_0087354 were consistent with those of microarray detection, indicating reliability of results of circRNA chips. There are positive correlation between the expression of hsa_circ_0038646 and ALT. These results indicate significant differences in circRNA expressions in PBMC at different stages of chronic HBV infection. Differences in circRNA expression may be related to mechanism of immune dysfunction in CHB patients.

circRNAs can act as miRNA sponge and regulate miRNA function by enriching miRNA binding sites [17]. Results predicted with Arraystar's miRNA software showed that differentially expressed circRNAs with MRE can pair with miRNAs on seed regions of complementary

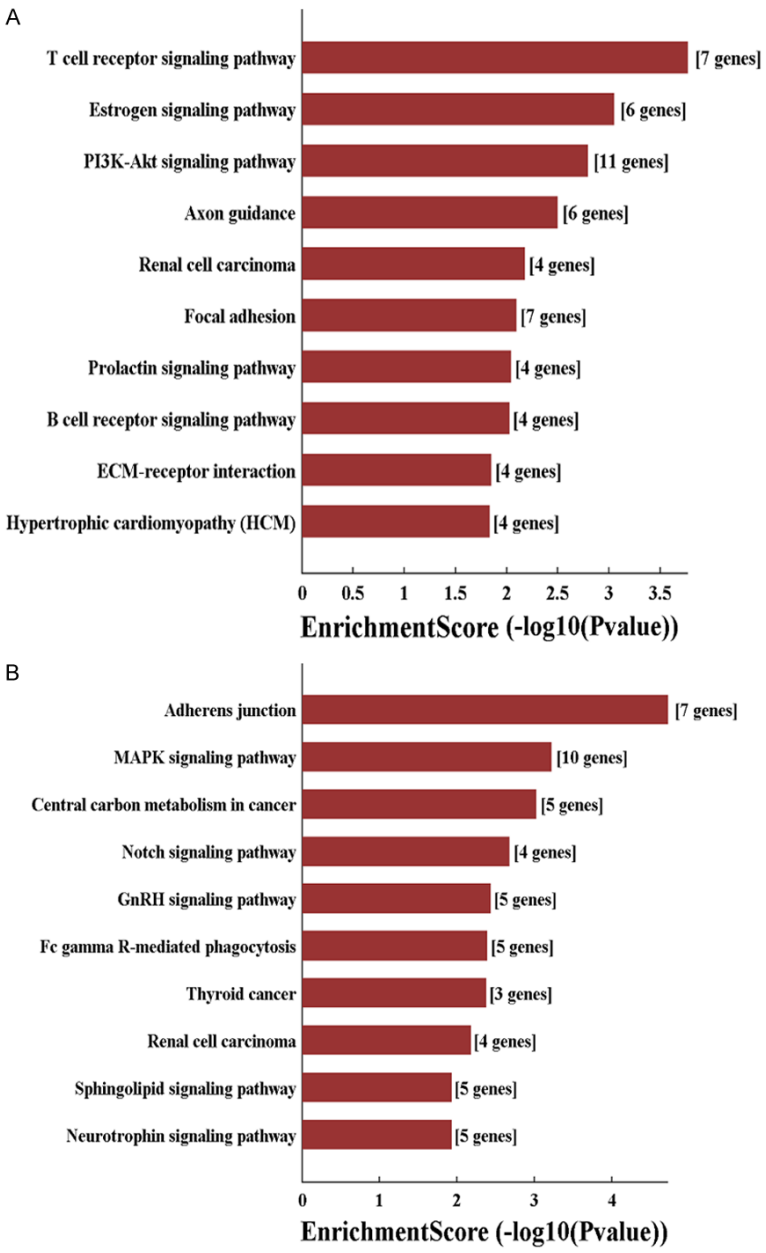

Figure 5. Pathway analysis maps of hsa_circ_0038646 and 0087354 target genes. A. hsa_circ_0038646; B. hsa_circ_0087354.

bases. A circRNA can be paired with various complementary miRNAs. A variety of circRNAs can be paired with complementary miRNAs. For example, hsa_circ_0038646 can be paired with complementary hsa-miR-181b-5p, hsa-miR-181c-5p, hsa-miR-181d-5p and hsa-miR-181a-5p. Hsa-miR-181c-5p and hsa-miR-181a-5p play key roles in the development and differentiation of T cells. Hsa-miR-181c-5p can regulate degree of inflammation [18-20]. Ng *et al*. [21] reported that knockdown of expression of mcircRasGEF1B, a *lipopolysaccharide* (LPS)-inducible circRNA, reduces LPS-induced intercellular adhesion molecule 1 (ICAM-1) expression and determined that mcircRasGEF1B regulates stability of mature ICAM-1 mRNAs. Ng *et al.* [18] further suggested that mcircRasGEF1B may play a critical role in fine-tuning immune responses and protecting cells against microbial infection. This study predicted the target genes regulated by miRNA-related hsa_circ_0038646 and hsa_circ_0087354. Results showed target genes of hsa_circ_0038646 miRNA-related regulation. A total of 533 target genes of hsa_circ_0087354 and 249 target genes of hsa_circ_0038646 were related to miRNA. These results indicate that differential expression of circRNAs in PBMC of CHB patients may play their regulatory function by targeting a number of genes. Specific regulation of target genes requires further research.

Molecular function and signaling pathway of target genes of hsa_circ_0038646 and hsa_circ_0087354 were analyzed by GO and pathway enrichment analyses. Results showed that functions of target genes regulated by miRNA-related hsa_circ_0038646 were mainly enriched in activin binding, S100 protein binding, GDP dissociation inhibitor activity, transforming growth factor receptor activity, and ubiquitin protein ligase gene activity. Molecular functions of target genes regulated by miRNA-related hsa_circ_0087354 were enriched in armadillo repeat domain binding, pre-mRNA binding, MAPK activity, translation repressor activity, and GTP-dependent protein binding gene activity. Results of pathway enrichment analyses of different target genes

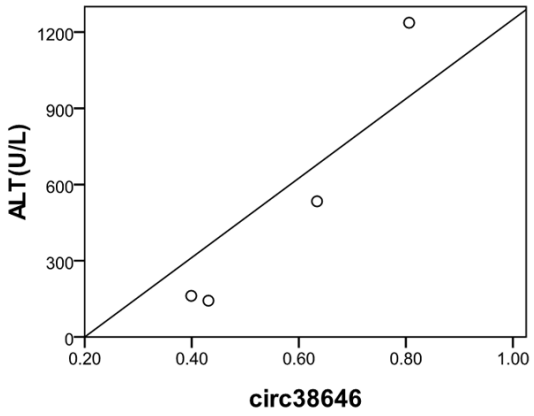

Figure 6. Scatter plot between hsa_circ_0038646 and ALT.

showed that miRNA-related hsa_circ_0038646 were mainly involved in T cell receptor binding pathway, estrogen receptor signaling, PI3K/Akt signaling, and axon guidance signals. Signaling pathways of miRNA-related hsa_circ_0087354 were mainly involved in adherens junction, MAPK signaling pathway, central carbon metabolism in cancer, Notch signaling pathway, and GnRH signaling pathway. These results indicate that hsa_circ_0038646 and hsa_circ_0087354 may regulate multiple gene-related signaling pathways and influence immune responses in chronic HBV-infected individuals.

In conclusion, this study showed significant differential expression of circRNAs in PBMC at different stages of CHB patients. Differential expression of circRNAs and their related miRNA may occur through regulation of multiple target genes and signaling pathways involved in immune regulation of chronic HBV infection.

Acknowledgements

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Disclosure of conflict of interest

None.

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