Original Article
Differential expression and bioinformatic analysis of circRNA in nonalcoholic steatohepatitis cirrhosis

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Abstract: Aim: This study investigates the expression profile of circRNA in nonalcoholic steatohepatitis (NASH) cirrhosis and identifies the underlying pathogenesis of core genes of NASH cirrhosis. Methods: The GEO 134146 dataset was obtained from GEO database. EdgeR software was used to analyze the differential expression of circRNA between NASH cirrhosis samples and normal samples, and Starbase and miRWalk databases were used to predict the targeted miRNA and mRNA. The protein-protein interaction network of these target genes was established by searching the string database of interacting genes, Cytoscape and Mcode analysis. In addition, David and Omicshare were used to analyze the functional enrichment and pathway enrichment of target genes. Results: We evaluated 99 differentially expressed circRNAs, 27 of which were up-regulated, and 72 were down-regulated. A regulatory network consisting of 10 circRNAs, 30 miRNAs, and 1217 mRNAs was further constructed. The differential expression of circRNA is closely related to the functions of “target gene transcriptional regulation”, “protein binding”, “serine/threonine kinase”, etc. The difference in circRNA is mainly related to the “MAPK” signaling pathway and the “FoxO” signaling pathway. Conclusions: This study confirmed the abnormal regulation of circRNA in NASH cirrhosis. Bioinformatic analysis showed that abnormal expression of circRNA might be related to the occurrence and development of NASH cirrhosis.

Keywords: Bioinformatics, circRNA, NASH, cirrhosis

Introduction

Nonalcoholic fatty liver disease (NAFLD) is caused by various conditions, especially obesity and eating habits. Excess accumulation of fat in human liver cells causes abnormal metabolism. At present, it has become a global chronic liver disease that endangers health. According to an American epidemiologic survey of chronic liver disease, 20%-30% of the population suffers from NAFLD [1]. NAFLD and its subtype non-alcoholic steatohepatitis (NASH) are an increasing clinical and economic burden for public health [2]. NASH is considered the aggressive form, with liver inflammation and hepatocyte injury in the form of ballooning. NASH also leads to liver fibrosis, and liver fibrosis is a key prognostic factor in evaluating liver-related diseases [3, 4]. Fibrotic NASH is a serious disease that requires active medical management with different drugs [5]. Drugs that target the pathogenesis of NAFLD are under rapid development. Unfortunately, liver biopsy is an invasive method for diagnosing NAFLD/early NASH, and NASH/liver cirrhosis, and is currently the most reliable method. Because patients are reluctant to undergo several biopsies, and the screening failure rate for including patients in clinical trials is high, biopsy histology is needed to determine which patients need treatment and to assess their response to treatment, thereby slowing the search for effective treatments for NAFLD [6]. NASH cirrhosis is a significant risk factor for liver cancer. Therefore, finding a non-invasive biomarker is significant for the diagnosis and treatment of NASH cirrhosis [7, 8].

CircRNA is a kind of special non-coding RNA molecule. It is composed of exon transcripts, which are formed by nonlinear reverse splicing, and circRNA molecules contain introns. Most
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circRNAs are covalently linked to each other by 3', 5'-phosphodiester bonds, without a polyadenylated tail [9]. Studies show that circRNA plays an active role in the occurrence and development of diseases [10]. Because it is not sensitive to nucleases, it is relatively stable, so circRNA has obvious advantages as a clinical diagnostic marker. Studies have found that more than 1,000 circRNAs are significantly enriched in human serum exosomes, and the expression level of circ-KLHDC10 has successfully distinguished between colorectal cancer patients and normal people [11]. circRNA also contains miRNA response elements; these elements can be used as competitive endogenous RNA (CeRNA), combined with miRNA to eliminate miRNA's inhibitory effect on target genes. Jin et al [12] found that circrna_002581 can regulate the expression of CPEB1 through miR-122. CPEB1 regulates autophagy through the PTEN-AMPK-mTOR pathway and participates in the development of NASH, indicating that it targets circrna_002581 which is a potential therapeutic target for NASH. This study explores the expression profile of circRNA in NASH cirrhosis, determines the potential role of hub genes in NASH cirrhosis, and further screens circRNA as a biomarker for NASH cirrhosis.

Materials and methods

Gene chip data acquisition

GSE134146 gene expression data derived from the GEO database (http://www.ncbi.nlm.nih.gov/geo) were from an expression profiling by array study type performed on a GPL21825 platform (074301 Arraystar Human CircRNA microarray V2). The GSE134146 data set included data on human HSCs, in which 4 cases of liver fibroblasts from NASH cirrhosis liver (n=4) and 4 cases of liver fibroblasts from the normal liver (n=4).

Identification of differentially expressed circRNAs

On the basis of circRNA expression, differential expression analysis was performed using the quasi-likelihood F test method of the edgeR [13] software package. The differential expression of liver fibroblasts from patients with NASH cirrhosis and normal human liver fibroblasts was screened, and the threshold was determined to be >1.0 (|logFC|>1), P value <0.01. The analysis requires circRNA expressed at a value greater than 0 in at least two samples, and unqualified circRNA is deleted. Omicshare tools was used to make volcanic and heat maps.

Construction of CircRNA-miRNA-mRNA network


The establishment and module analysis of PPI network

Using the string [19] database (http://www.string-db.org/), the association between known proteins and predicted proteins can be retrieved and used to predict protein-protein interaction (PPI) information. The target genes regulated by differential circRNA were imported into string database to obtain the data for target gene interaction. Cytoscape3.5.0 [20] (http://www.cytoscape.org/) can be used to display the relationship between target genes. The resulting PPI network module can be analyzed by mcode [21] clustering algorithm in Cytoscape. Modular analysis can be used to identify other linkage genomes.

Bioinformatics analysis of differentially expressed circRNA

The differentially expressed circRNA host genes were analyzed by David tool (v6.8) [22] to analyze their rich GO functions and KEGG pathway. The results of significant enrichment were considered as the parameter of enrichment gene count ≥2 and the significance threshold of the hypergeometric test was P<0.05.

Results

Differential expression of circRNA in NASH cirrhosis

There were 4 NASH cirrhosis samples and 4 normal samples in this study. According to cut-
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**A**

volcano_plot

![](volcano_plot.png)

**B**

![Heatmap](heatmap.png)
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Figure 1. Differential expression of circRNA between NASH cirrhosis and normal liver tissue. A. CircRNA volcanic plot. The horizontal axis represents the standardized difference, the vertical axis represents the normalized p value, and the red and green dots in the figure are the significant differentially expressed genes. B. The color green or red indicates that the expression of circRNA is low to high respectively. Each column on the map represents a sample, and each row represents a gene.

Table 1. 10 up-regulated and down-regulated circRNAs

<table>
<thead>
<tr>
<th>CircRNA ID</th>
<th>Expression</th>
<th>Position</th>
<th>Genomic Length</th>
<th>Gene symbol</th>
<th>CircRNA ID</th>
<th>Expression</th>
<th>Position</th>
<th>Genomic Length</th>
<th>Gene symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa_circ_0089761</td>
<td>Up</td>
<td>ChrM</td>
<td>8302</td>
<td>None</td>
<td>hsa_circ_0004662</td>
<td>Down</td>
<td>Chr6</td>
<td>5769</td>
<td>SOD2</td>
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<tr>
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<td>ChrM</td>
<td>5783</td>
<td>None</td>
<td>hsa_circ_0066745</td>
<td>Down</td>
<td>Chr3</td>
<td>74785</td>
<td>MYH15</td>
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<tr>
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<td>ChrM</td>
<td>396</td>
<td>None</td>
<td>hsa_circ_0039459</td>
<td>Down</td>
<td>Chr16</td>
<td>932</td>
<td>MT2A</td>
</tr>
<tr>
<td>hsa_circ_0088882</td>
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<td>ChrM</td>
<td>81</td>
<td>None</td>
<td>hsa_circ_0008213</td>
<td>Down</td>
<td>Chr20</td>
<td>776</td>
<td>NCOA6</td>
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<tr>
<td>hsa_circ_0075447</td>
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<td>Chr6</td>
<td>16171</td>
<td>GMDS</td>
<td>hsa_circ_0074206</td>
<td>Down</td>
<td>Chr5</td>
<td>2449</td>
<td>MATR3</td>
</tr>
<tr>
<td>hsa_circ_0043151</td>
<td>Up</td>
<td>Chr17</td>
<td>10056</td>
<td>MYO19</td>
<td>hsa_circ_0082720</td>
<td>Down</td>
<td>Chr7</td>
<td>57925</td>
<td>DENND2A</td>
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<tr>
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<td>Chr3</td>
<td>2019</td>
<td>TKT</td>
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<td>Down</td>
<td>Chr10</td>
<td>86145</td>
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<tr>
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<td>61963</td>
<td>HIPK2</td>
<td>hsa_circ_0016169</td>
<td>Down</td>
<td>Chr1</td>
<td>12106</td>
<td>None</td>
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<tr>
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<td>Up</td>
<td>Chr7</td>
<td>11493</td>
<td>None</td>
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<td>Down</td>
<td>Chr8</td>
<td>112157</td>
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<td>5053</td>
<td>NCOA7</td>
</tr>
</tbody>
</table>

Figure 2. The ceRNA network of circRNA-miRNA-mRNA in GC. The red circle represents circRNA, the pink triangle represents miRNA, and the blue diamond represents mRNA.

off criteria |logFC|>1, P value was <0.01. Compared with the normal group, there were 99 differentially expressed genes in the NASH cirrhosis group (27 up-regulated genes and 72 Down-regulated genes), see Figure 1A volcano plot. Figure 1B heatmap shows the expression of genes in different groups. We screened the top 10 differentially expressed up- and down-regulated circRNAs, see Table 1.

The networks of circRNA-miRNA-mRNA identification

We used the first 10 up-regulated and down-regulated circRNA with a significant differential expression for further analysis, and used 14 circRNAs that could be identified by both Starbase and circinteractome databases to predict targeted miRNAs. Finally, 30 miRNAs
were predicted by 10 circRNA in the two databases for further analysis. The miRWalk, miRbase, and targetscan databases were used to predict miRNA targeted mRNA. A total of 1217 target genes were obtained from the common prediction results of the three databases. The regulatory network of circRNA-miRNA-mRNA is shown in Figure 2.

**Target gene functional enrichment analysis**

Enrichment analysis of GO function and KEGG pathway were performed on circRNA host genes. According to P<0.01, we enriched 52 biologic processes (BP), 21 cell components (CC), 27 molecular functions (MF), and 52 KEGG pathways. Differentially expressed circRNA is
related to the transcriptional regulation of target genes (Figure 3A), is mainly located in the nucleus and cytoplasm and on the membrane of organelles (Figure 3B), which can bind to proteins, and has serine and threonine kinase activity (Figure 3C). "MAPK" signaling pathway and "FoxO" signaling pathway were meaningful pathways (Figure 3D). These two approaches are shown in Figure 4.
Target gene interaction analysis and mcode analysis

Using the string database to obtain the PPI network graph of 871 nodes and 4056 edges (Figure 5A), the Mcode analysis method could identify the core genes in the PPI network. According to the screening conditions where K-core is equal to 2 and score is greater than 7, we obtained 3 modules, scored 18 (Figure 5B), 10.486 (Figure 5C), 7.12 (Figure 5D). The 18 genes contained in the module with the highest score RNF213, RNF41, RNF114, CBLB, RNF144B, FBXL19, FBXL18, UNKL, KLHL2, RNF19B, KLHL5, UBE2H, CUL3, UBE2W, UBE2G1, UBE2V1, UBE3A, RLIM are considered to be hub genes. Based on this hub gene, we constructed a circRNA-miRNA-mRNA network (Figure 6). This molecular regulatory network should be the key mechanism for the regulation of NASH cirrhosis. We used the Cytoscape software ClueGO plug-in to enrich and analyze the three selected module genes. Its functions mainly involve the regulation of transcription signals, the regulation of vascular endothelial cell function, cellular response to oxidative stress, and tissue reconstruction (Figure 7).

Discussion

CircRNA was discovered as early as 1976, and in 1979 was also found to be the endogenous RNA splicing product of eukaryotes [23]. With the development of RNA sequencing technology and bioinformatics, the richness and diversity of circRNA have been identified, and the changes in the expression patterns of circRNA under different developmental stages and physiologic conditions have been revealed. Many
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Figure 6. The circRNA-miRNA-mRNA network of hub genes. Blue circles represent mRNA, green diamonds represent miRNA, and red triangles represent circRNA.

circRNA functions have also been discovered, such as serving as a scaffold in the assembly of protein complexes [24], separating proteins from their inherent subcellular locations [25], regulating parental gene expression [26], regulating alternative splicing, and RNA protein interaction [27]. It also functions as a miRNA sponge. It has been proven that circRNA plays an important role in the pathogenesis of diseases [28].

NAFLD is the accumulation of abnormal fat in the liver. Without proper control, NAFLD may develop into NASH, which is more serious than NAFLD. Especially in the western world, the incidence of NAFLD and NASH have increased owing to lifestyle changes and modern diet. In addition, these liver manifestations are highly correlated with cirrhosis and liver cancer [29, 30]. Cirrhosis is the stage of liver scar formation, which can be induced by different liver injury factors. With the formation of scar tissue, it is difficult for the liver to function normally. Generally, the damage caused by severe cirrhosis is irreversible. Early diagnosis and intervention may limit further damage caused by cirrhosis. Liver cancer has high mortality and mainly occurs in patients with chronic hepatitis or cirrhosis. NASH and NASH-related cirrhosis are high risk factors for liver cancer. Unfortunately, liver cancer treatment is often less effective. Therefore, it is urgent to understand the molecular mechanism of NASH cirrhosis [31, 32]. circrna_0046367 can regulate the expression of miR-34a, which is a kind of miRNA promoting steatosis. A normal level of circrna_0046367 can improve lipid peroxidation, apoptosis, and mitochondrial dysfunction in steatosis, and eliminate the inhibitory effect on miR-34a, indicating circrna_0046367 can improve lipid toxicity and oxidative stress in NAFLD [33]. Circ_0071410 down-regulation can increase the expression of miR-9-5p and further reduce the activation of hepatic stellate cells. These data indicate that circRNA can regulate the activation of hepatic stellate cells in the liver [34]. Therefore, basic experiments are needed to clarify the role of circRNA in NAFLD/NASH-related liver fibrosis/cirrhosis.

By cluster analysis, we found that significant differences in the expression of circrna between NASH cirrhotic tissues and normal tissues. The heat map indicates that circRNA expression in NASH cirrhosis is significantly up-regulated or down-regulated compared to normal tissues. This suggests that there are significant differences in the expression of certain circular RNAs during the onset of NASH cirrhosis. Bioinformatic analysis of the differential genes indicates that the differential expression of circRNA may be related to protein binding, serine/threonine kinase activity, and target gene transcriptional regulation. KEGG pathway analysis found that the “MAPK” signaling pathway and “FoxO” signaling pathway may be closely related to NASH cirrhosis.

MAPK is an important member of the MAPKs family that regulates inflammation. It can promote the aggregation and activation of leuko-
cytes, regulate the activity of transcription factors, and regulate the synthesis of cytokines such as TNF-α, IL-1, IL-6, and other inflammatory factors. The regulation of this response plays a key role [35]. Studies have found that MAPK in macrophages can regulate the release of inflammatory cytokines and regulate the macrophage M1 polarization to promote steatohepatitis [36]. FoxO participates in the formation of NASH by regulating the metabolism of lipid and glucose in the liver [37]. FoxO can also participate in regulating inflammatory responses in the body by regulating the expression of interleukin-1β and toll-like receptor 4 and other inflammatory factors. Mice lacking FoxO1/3/4 in their bodies are more likely to undergo inflammation and liver damage stimulated by high-fat and cholesterol diets [38].

The circRNA-miRNA-mRNA network of NASH cirrhosis has been established in this study. This helps to further understand the role of circRNA in NASH cirrhosis. We screened the hub gene according to the Mcode algorithm. We established a sub-circRNA-miRNA-mRNA network, which allowed us to understand the core molecular regulatory mechanism of NASH cirrhosis. Among them, the circ_0089761 molecule can be used as a biomarker for NASH cirrhosis for noninvasive examination provides a theoretical basis, and its role in NASH cirrhosis needs further study. The selected module genes were further subjected to functional annotation analysis and found to be mainly concentrated in "regulation of tissue structure reorganization", "regulation of vascular endothelial cell physiological function", "response to oxidative stress", and "regulation of cell death". These functions are closely related to NASH cirrhosis, indicating that these molecules can be used as target molecules for drug therapy.

In conclusion, this study determined a differential expression of circRNA in NASH liver cirrhosis. Bioinformatic analysis shows that the abnormality of circRNA may be closely related to the disease process.
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to the occurrence and development of NASH liver cirrhosis. This study lays a foundation for further study on the molecular regulation mechanism of circrna in NASH cirrhosis.

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Disclosure of conflict of interest

None.

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