Differential Expression of MicroRNAs in Fibrotic Liver Tissues of Patients with Chronic Hepatitis B

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Abstract

Background: MicroRNAs (miRNAs), which are single-stranded small RNAs approximately 21 to 23 bases long, are involved in the regulation of cell proliferation, apoptosis, and lipid metabolism/fatty acid metabolism, and play important roles in the differentiation of the liver and the maintenance of its morphology and function. MiRNAs are also closely related to the occurrence, development, treatment, and prognosis of liver diseases.

Fibrotic Liver is a chronic process of the intrahepatic damage–repair response. The mixed effects of these pathogenic factors result in an abnormal comprehensive proliferation of fibrous tissue in the liver, diffused production and deposition of the extracellular matrix (ECM), and an imbalance in ECM synthesis and degradation. These lead to secondary inflammatory responses in the liver and the reversible pathological process of self-healing.

Methods: Liver tissues were obtained from 3 patients with CHB with liver histopathological fibrosis grade ≥ S2, 2 patients with CHB with fibrosis grade < S2, and 1 healthy individual with a normal liver. The high-throughput miRNA microarray technique was used for miRNA expression analysis. The screening criteria for differential miRNA expression were a fold-change ≥ 2 and \( P < 0.005 \). The miRNAs with significantly different expression levels were verified by quantitative real-time PCR (qPCR), and the target gene functions were predicted by Gene Ontology (GO) and pathway analyses. Finally, an miRNA-gene network map was constructed.

Results: In the CHB groups of tissues, 34 miRNAs with ≥ 2-fold difference in expression were found, of which 18 were upregulated and 16 were downregulated. qPCR was used to verify these miRNAs that showed significant differences, with the results showing consistency with the microarray results, indicating that the miRNA microarray results were credible. Bioinformatics analysis results demonstrate that some of the key upregulated miRNAs found in the network map were hsa-miR-125b-2-3p, hsa-miR-4639-3p, hsa-miR-4764-3p, and hsa-miR-3133, whereas some of the key downregulated ones were hsa-miR-1297, hsa-miR-154-5p, hsa-miR-3183 and hsa-miR-663a.

Conclusion: In patients with CHB, the miRNA expression profile changes significantly with the severity of fibrotic liver. The development of fibrotic liver may be related to the miRNA-mediated regulation of cell development, metabolism, and apoptosis, and the positive/negative regulation of cell processes.

This study was approved by the ethics committee of general hospital of Ningxia medical university (2015-134).

Background

MicroRNAs (miRNAs), which are single-stranded small RNAs approximately 21 to 23 bases long, are involved in the regulation of cell proliferation, apoptosis, and lipid metabolism/fatty acid metabolism, and play important roles in the differentiation of the liver and the maintenance of its morphology and
MiRNAs are also closely related to the occurrence, development, treatment, and prognosis of liver diseases. Fibrotic Liver is a chronic process of the intrahepatic damage–repair response. The mixed effects of these pathogenic factors result in an abnormal comprehensive proliferation of fibrous tissue in the liver, diffused production and deposition of the extracellular matrix (ECM), and an imbalance in ECM synthesis and degradation. These lead to secondary inflammatory responses in the liver and the reversible pathological process of self-healing.

The central link in the development of hepatic fibrosis is the activation and proliferation of hepatic stellate cells. MicroRNAs can cooperate with cytokines like platelet-derived growth factor, transforming growth factor (TGF), and peroxisome proliferator-activated receptor to regulate liver fibrosis. For example, miRNAs have been shown to promote hepatic stellate cells activation, proliferation, or apoptosis, thus mapping into a complex network of genes that regulate fibrosis. This suggests that miRNAs may be involved in the regulation of the biological behavior of hepatic stellate cells and affect the occurrence and development of liver fibrosis. MicroRNAs associated with liver fibrosis are mainly related to chemical toxicant damage (carbon tetrachloride, dimethyl nitrosamine, etc.), immune damage response (injection of heterologous proteins, etc.), and cholestasis. However, whether liver fibrosis-associated miRNAs found in the tissue and plasma of murine models with chronic hepatitis B (CHB) can fully explain the development of liver fibrosis in human patients with CHB needs further study.

The expression of miRNAs in the liver tissue of patients with CHB with advanced liver fibrosis is still rarely reported in China and abroad. Therefore, this study used high-throughput sequencing technology to analyze the differential expression and preliminary functions of miRNAs in fibrotic liver tissues in patients with CHB, with the aims being to reveal the mechanism of fibrotic liver and to provide a basis for future research on the prevention and treatment of the condition.

**Methods**

**Subjects**

Four normal control liver tissues were selected from the patient with liver repair surgery because of trauma from a car accident in department of hepatobiliary surgery of General Hospital of Ningxia Medical University from May 2014 to January 2017. Twenty-six patients with CHB (11/26 were patients only with CHB, 15/26 were patients with CHB and liver cirrhosis) confirmed by clinical and laboratory examinations at the outpatient clinic and inpatient department of General Hospital of Ningxia Medical University from May 2014 to January 2017 were selected. The following were the inclusion criteria: (1) All patients with CHB had complete medical history data and had been classified according to the clinical diagnostic criteria of the 2015 Guidelines for the Prevention and Treatment of Chronic Hepatitis B; (2) All patients had liver function, hepatitis B virus (HBV) serological markers, serum HBV DNA, and liver biopsy results;
(3) All patients were not treated with liver protection, anti-fibrosis, and antiviral drugs before enrollment. The following were the exclusion criteria: (1) HBV-carrying patients with immune tolerance and overlapping infections, such as hepatitis A virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, etc; (2) Liver damage-causing factors, such as violence, trauma, autoimmunity, drugs, and alcohol consumption. In addition, normal liver tissue samples were collected from 4 healthy volunteers. All serum samples were from the Department of Infectious Diseases, General Hospital of Ningxia Medical University. The relevant data of the patients are shown in table S1. All respondents were informed about the nature of the study and signed informed consent forms before the survey was taken. The liver tissue samples were stored at -80 °C until use. This study was approved by the ethics committee of general hospital of Ningxia medical university (2015 − 134).

**Extraction and quality detection of total RNA from the liver tissue samples**

Total RNA in the tissue samples was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. The $A_{260}$ and $A_{280}$ absorbance values of the total RNA were then determined using a UV spectrophotometer. The concentration of the RNA was calculated from the $A_{260}$ value, and the purity from the $A_{260}/A_{280}$ ratio. The quality of the total RNA was further verified by denaturing formaldehyde agarose gel electrophoresis.

**MicroRNA microarray detection of miRNA expression in the different liver tissues**

The profiling of miRNA expression was conducted using miRNA microarray technology and was carried out by KangCheng Bio-Tech (Shanghai, China). In order to reduce the influence of sex, age, and basic disease in all the liver tissue specimens, the samples from 1 individual with a normal liver, 3 patients with CHB, and 2 patients with hepatitis B cirrhosis (all aged 30−40 years old) were evaluated using the LNA miRNA chip (version 18.0) produced by Exiqon (Vedbæk, Denmark), excluding other basic diseases. This chip uses a capture probe that is based on the patented locking nucleic acid (LNA) technology, which is highly sensitive and specific. The probe binds specifically to complementary target RNA to accurately detect miRNA expression levels in the sample. Each miRNA chip contains at least 1807 specific probes and 435 Exiqon-specific miRPlus probes (providing new miRNA information outside of the miRBase database), control probes, and probe-free blank controls, and can detect human, mouse, and rat miRNAs in the Sanger miRBase 21.0 database (containing ~ 847 human, 609 mouse, and 351 rat miRNAs). To ensure the reliability of the results, each of the above probes was repeated 4 times in the chip; that is, each chip was repeatedly tested 4 times for the same sample. In brief, 5 µg of total RNA from the 3 different types of liver tissue samples described above were first labeled with the fluorescent dye Hy3, using the miRCURY Array Labeling Kit (Exiqon). The RNeasy Mini Kit (Qiagen, Hilden, Germany) was then used to concentrate the labeled samples, following which miRNA chip hybridization was performed using the miRCURY Array Microarray Kit (Exiqon) and Hybridization Chamber II (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). The specific steps were performed according to the manufacturers’
instructions. The chip was scanned with a GenePix 4000B device (Molecular Devices, San Jose, CA, USA) at 635 nm and the data were analyzed using GenePix Pro 6.0 software (Molecular Devices). The green signal intensity of each probe on the chip was background corrected, and 4 replicate probes were taken. The data were normalized by the median standardization method and the standardized data were obtained. The non-control probes with correction values (foreground value minus background value) of ≥ 50 on each chip were selected for standardization in a batch of experiments. This part of the probe median is used as a normalization factor to normalize the points of the entire chip; that is, each miRNA correction value/median value = standard value. After standardization, the differentially expressed miRNAs were tested by the statistical T-test method. Unsupervised clustering and correlation analyses of the miRNA microarray data were also carried out.

Real-time quantitative PCR detection of target miRNA expression

The miRNA expression levels in the 6 above-mentioned liver tissue samples were detected in 30 samples (4 normal control liver tissues, 11 liver tissues with chronic hepatitis B, 16 liver tissues with chronic hepatitis B and liver cirrhosis) using real-time quantitative polymerase chain reaction (qPCR). In brief, 2 µg of total RNA from each tissue sample was used as the initial template, in a total reaction mixture of 20 µL, for the reverse transcription synthesis of cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA). The reaction was carried out on an Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: 16 ℃ for 30 min; 42 ℃ for 40 min; and 85 ℃ for 5 min. Then, using 1 µL of the cDNA as a template (in 3 replicate wells per test sample), qPCR was performed on a Rotor-Gene 3000 Real-time PCR device (Corbett Research, Cambridge, UK), with U6 used as an internal reference. The reaction conditions were as follows: preheating at 95 ℃ for 10 min; 35 cycles of 95 ℃ for 10 s, 60 ℃ for 60 s, and 95 ℃ for 15 s; and 54 cycles of 0.5 ℃ for 5 s. The primers used in the PCRs were synthesized by Invitrogen. To reduce the effects of quantitative errors in RNA concentration and efficiency errors in RNA reverse transcription, the obtained data were corrected with the 2-ΔΔCt formula. The corrected values were then used to determine the differences in the expression levels of the target miRNAs in each sample.

Bioinformatics and microarray data analysis

All the GO terms in which the genes were involved were obtained, and the categories were then tested with Fisher’s exact test and the chi-square test. The significance level and false-positive rate of each GO term were calculated to screen out the significant ones reflected by the target genes, with the criteria for significant screening being P < 0.01. The ordinate is the signal path or biological process, and the abscissa is the enrichment value (Enrichment Score), which is calculated by -log10(P value). The larger the value is, the smaller the P value will be, indicating that the signal path is more significant.

Statistical analysis

All the data statistical analysis was performed by SPSS23.0 software (IBM, USA) and GraphPad Prism version 8.0 (GraphPad Software, USA), the data were presented as mean ± SD. The continuous variables
were tested by the t-test. Differences were considered significant if p < 0.05 (* p < 0.05; **p < 0.01).

Results

Quality of the total RNA

According to the UV spectrophotometric analysis, the $A_{260}/A_{280}$ values of the total RNA in the 5 liver tissue samples (i.e., from patients with CHB and hepatitis B cirrhosis) were 1.89, 1.86, 1.80, 1.84, and 1.89, respectively. Gel electrophoresis of the total RNA indicated clear 18S and 28S bands. These results indicated that the total RNA of these liver tissue samples from patients with CHB-related Fibrotic Liver Tissues had good quality and completeness and met the experimental requirements of the subsequent miRNA chip detection and qPCR (Fig. 1).

miRNA chip test results

The microarray results showed that 34 miRNAs were differentially expressed in patients with hepatitis B cirrhosis than patients only with CHB. With the filter criteria as fold-change $\geq$ 2, 18 were upregulated and 16 were downregulated (Fig. 2, Table S2).

To further verify the candidate miRNA, we use the filter criteria as fold-change $\geq$ 2, P-value < 0.05, Fluorescence value $\geq$ 100. The 8 candidate miRNAs were selected included 4 upregulated miRNAs: hsa-miR-125b-2-3p, hsa-miR-4639-3p, hsa-miR-4764-3p, and hsa-miR-3133, 4 downregulated miRNAs, hsa-miR-519d-3p, hsa-miR-1297, hsa-miR-3183, hsa-miR-154-5p, and hsa-miR-663a.

Target miRNAs determined by real-time quantitative PCR

To verify the 8 selected miRNA, 30 samples (4 normal control liver tissues, 11 liver tissues with chronic hepatitis B, 16 liver tissues with chronic hepatitis B and liver cirrhosis) were used in an independent cohort. RT-qPCR proved that the expression of 8 selected miRNA was consistent with the gene microarray results. (Fig. 3). The results suggested that these 8 selected miRNAs may play some important role in patients with CHB progressing to hepatitis B cirrhosis

Gene Ontology of the target genes of the miRNAs

In order to find out which genes may be related to the differentially expressed miRNAs, the target genes were screened using the Gene Ontology (GO) database for annotation of their functions. The target genes corresponding to the top 10 upregulated miRNAs in total of 1552 significant functions (Fig. 4-A), including negative regulation of cellular processes, regulation of primary metabolic processes, regulation of molecular metabolic processes, and positive regulation of nitrogen complex metabolic processes. The target genes of the top 10 downregulated miRNAs in total of 1391 significant functions (Fig. 4-B), including the development of multicellular organisms, the development of anatomical structures, the process of protein modification, and the positive regulation of cellular processes.
Pathways of the target genes

Pathway analysis of the identified target genes was carried out next. For the target genes of the upregulated miRNAs, 27 significant signaling pathways were found (Fig. 5-A), including the calcium signaling pathway, the VEGF signaling pathway, the insulin signaling pathway, and the ABC transporter receptor. For the target genes of the downregulated miRNAs, 76 significant signaling pathways were identified (Fig. 5-B), including the MAPK signaling pathway, the cancer-related pathway, the intracellular tropism pathway, the axon guidance pathway, and the TNF signaling pathway.

Regulatory networks for the differentially expressed miRNAs and overlapping target genes

To identify the target genes and their key processes and regulatory pathways, network analysis was used to view how the key components of the different pathways interact. The miRNA-target genes network map for the upregulated and downregulated miRNAs are shown in Figs. 6 respectively. The key miRNAs found to be upregulated included hsa-miR-550a-3p, hsa-miR-3133, hsa-miR-125b-2-3p, hsa-miR-4275, hsa-miR-421, hsa-miR-374b-3p, and hsa-miR-574-3p. The key target genes for regulation included NM_55183, NM_6908, NM_2969, and NM_4214. The key downregulated miRNAs included miR-519d-3p, miR-3183, miR-938, miR-138-5p, and miR-1297. The key target genes for regulation included NM_1285, NM_369, NM_80223, NM_10564, NM_10087, NM_6416, and NM_2321.

Discussion

MicroRNAs are an important class of epigenetic regulators, researching their relationship with fibrotic liver would be of great significance to elucidating the mechanism of the condition. In this study, 34 miRNAs were differentially expressed by ≥ 2 fold (P< 0.005) in the liver tissue of patients with CHB-associated fibrotic liver compared with that in patients with liver fibrosis grade ≥ S2. Of these 34 miRNAs, 18 were upregulated and 16 were downregulated. Some of the miRNAs were verified by qPCR. Following further screening based on the criteria of a differential expression multiple of ≥ 2 times, P< 0.005, and the original signal RI value being larger, 9 miRNAs were selected, of which 4 were upregulated (viz., hsa-miR-125b-2-3p, hsa-miR-4639-3p, hsa-miR-4764-3p, and hsa-miR-3133) and 5 were downregulated (viz., hsa-miR-519d-3p, hsa-miR-1297, hsa-miR-3183, hsa-miR-154-5p, and hsa-miR-663a). qPCR verified that these 9 miRNAs were consistent with the chip results and that the data were credible. Other miRNA chip results are pending further validation.

The miRNA hsa-miR-3133 screened in this study has been confirmed to be involved in the formation of fibrotic liver. Peng found that miR-3133 could downregulate TGF-β (and its receptor TGFBR1) that promotes liver fibrosis in vivo. MiR-3133 downregulated might be an independent prognostic biomarker in ccRCC patients and plays inhibitory roles in aggressive progression of ccRCC. At the same time, studies have shown that miR-3133 can increase the expression of the recombinant adeno-associated
virus by downregulating the ErbB signaling pathway that involves its target gene CBL, thereby improving the expression efficiency of this gene therapy vector for hepatic fibrosis.

Some of the differentially expressed miRNAs found in this present study, such as hsa-miR-125b-2-3p, hsa-miR-4639-3p, hsa-miR-4764-3p, hsa-miR-1297, hsa-miR-3183, hsa-miR-154-5p, hsa-miR-663a, and hsa-miR-519d-3p, have not been reported to be related to fibrotic liver, but they have been reported in other diseases. For example, hsa-miR-125b-2-3p was found to regulate prostaglandin F2α receptor inhibitors by regulating its target gene PTGFRN, which is closely related to the metastatic status of osteosarcoma. Studies have also shown that miR-125b-2-3p may be involved in the inflammatory response following cerebral ischemic injury and is closely related to the pathophysiology of the condition\textsuperscript{16}. miR-4639-3p is currently reported to be associated with the development of early-onset familial Alzheimer's disease, and its high expression in the plasma of patients with chronic gout may be associated with gout formation\textsuperscript{17}. Recent studies have shown that hsa-miR-154-5p may play a role in smoking-induced lung cancer through the regulation of its target genes (ACTB, ATP2A2, BDNF, and CUL2) and the HIF-1, MAPK, Notch, and autophagic molecular signaling pathways and autophagic molecules\textsuperscript{18}. Some scholars have suggested that hsa-miR-154-5p could contribute to the antitumor activity of vitamin D in prostatic hyperplasia through direct or indirect targeting of the stromal-epithelial crosstalk gene during the progression of prostate cancer\textsuperscript{19}. miR-663a is associated with the progression of endometrial carcinoma\textsuperscript{20}. miR-519d-3p and miR-1297 can be used as tumor suppressor genes in cervical cancer\textsuperscript{21}. In addition, some of the miRNAs found in this study that are not related to the formation of fibrotic liver, such as hsa-miR-4764-3p, hsa-miR-3941, hsa-miR-4694-5p, hsa-miR-138-5p, hsa-miR-5571-5p, and hsa-miR-4421, are worth further study.

The GO analysis found that the differentially expressed miRNAs could regulate cell development, proliferation, and apoptosis, and may also regulate molecular metabolic processes, nitrogen complex metabolic processes, protein modification processes, and the positive regulation of cellular processes. The pathway analysis suggested that the differentially expressed miRNAs were mainly involved in proliferation- and apoptosis-related signaling pathways, as well as pathways related to microcirculation in fibrotic liver. The finding of these two types of signaling pathways is consistent with that of current literature reports. For example, proliferation- and apoptosis-related signaling pathways, such as the Wnt signaling pathway and TGF-β signaling pathway, have been reported to be closely related to the formation of hepatic fibrosis\textsuperscript{23–25}. In addition, our analysis also found a relationship between fibrotic liver and some other signaling pathways, such as the intracellular tropism pathway, axon guidance pathway, TNF signaling pathway, insulin signaling pathway, and the ABC transporter, which has not been reported before. Therefore, our study suggests that these miRNAs may regulate the progression of fibrotic liver through these pathways, although further experimental studies are needed to confirm this.

Using the graph theory method to evaluate the regulatory status of the miRNAs and genes in the network, it was found that the most significant gene in the network map may play the most significant role in the signaling pathway. The key targets of miRNA regulation were the genes NM_021843 (regulated by 7
microRNAs) and NM_012671 (regulated by 6 microRNAs). NM_053679 (DNA fragmentation factor subunit alpha (DFFA)) and NM_012775 (TGF-β1), the key target genes regulated by 5 downregulated miRNAs, were located at the center of the gene regulatory network. TGF-β1 is a member of the cytokine TGF-β superfamily, which is named for its ability to promote the transformation and growth of fibroblasts. The miRNA may target DFFA and TGF-β1 to regulate the signaling pathways involved in fibrotic liver.

**Conclusion**

In conclusion, the mechanism underlying the pathogenesis of fibrotic liver is very complicated. This study has found some new mechanisms related to fibrotic liver from the perspective of miRNAs, which not only further enriches our understanding of the pathogenesis of fibrotic liver from the perspective of miRNA regulation but will also aid research on the prevention and treatment of fibrotic liver in the future. Our study has important practical significance and academic value by virtue of providing a new direction and new ideas for early intervention strategies against fibrotic liver.

**List Of Abbreviations**

- Chronic hepatitis B (CHB)
- Chronic hepatitis B virus (HBV)
- Extracellular matrix (ECM)
- Hepatic stellate cells (HSC)
- MicroRNAs (miRNAs)
- Transforming growth factor (TGF)
- Fibrotic liver (FB)

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable

**Availability of data and materials**

Underlying raw sequencing data are not available due to privacy protection laws.

**Competing Interest**

This work does not have any relationships with business related issues, and no conflict of interest exits of all authors in the submission of this manuscript.
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Authors’ contributions
Yan-Li Zhang participated in all experiment, revised article, and coordinated all aspects of work, Xiang-Chun Ding: designed project, revised article, and coordinated all aspects of work, Long Hai: participated in all experiment, data analysis, create figures, wrote article, Li-Na Ma: subjects collected, inclusion criteria, Ying-Xia Zhao participated in experiment, create tables, Xia Luo: participated in experiment, data Cleaning, Xiao-Yan Liu participated in microarray data analysis, Yan-Chao Hu done the statistical analysis. Shah Nawaz has done the revision and editing.

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Figure 1

Gel electrophoresis of the total RNA. Lane 1: normal control; Lanes 2-4: chronic hepatitis B; Lanes 5-6: hepatic sclerosis
Figure 2

Diagram of the differentially expressed microRNA classes. A. heat map of differentially expressed microRNA; B. Histogram of differentially expressed microRNA
Figure 3

RT-qPCR verified differentially expressed miRNAs compared with MicroRNA microarray. 1: Normal control; 2: Chronic hepatitis B; 3: Hepatitis B cirrhosis; **P<0.05, **P<0.01 (3 vs 2).
Figure 4

Gene Ontology (GO) analysis of target genes. A. GO analysis the target genes of upregulated microRNAs; B. GO analysis the target genes of downregulated microRNAs
Figure 5

Pathway analysis of target genes in the liver cirrhosis group compared with microRNAs expression in the hepatitis group. A. Pathway analysis of upregulated microRNAs; B. Pathway analysis of downregulated microRNAs
Figure 6

The miRNA-target genes network map. A. Network analysis of upregulated microRNAs; B. Network analysis of downregulated microRNAs