Down-regulation of microRNA-31 suppresses hepatic fibrosis induced by carbon tetrachloride

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Abstract
MicroRNA-31 (miR-31) is among the most frequently altered microRNAs in human diseases, and altered expression of miR-31 has been detected in a large variety of diseases types. miR-31 could also regulate a variety of cell functions including hepatic fibrosis. Hepatic stellate cells (HSCs) are regarded as the major cell type involved in hepatic fibrosis. Male BALB/c mice (five mice per group aged 6 weeks) received 200 μL of body weight of carbon tetrachloride (10% CCl₄) mixed with olive oil intraperitoneally, and the first dose was doubled. To induce hepatic fibrosis, carbon tetrachloride was injected twice a week for 4, 6, 8, and 10 weeks. Control animals were injected with an equal volume of olive oil at the same time intervals. We found that miR-31 expression and fibrosis-related factors in four hepatic fibrosis stages. However, we noted that inhibition of miR-31 was down-regulated fibrosis-related factor expression in F1–F3 stages, but no F4 stage. Thus, we hypothesize that miR-31 may mediate hepatic fibrosis. In this research, we found that inhibition of miR-31 expression significantly inhibited HSC activation. The biological function of miR-31 during HSC activation might be through targeting hypoxia-inducible factor 1-alpha inhibitor (HIF1AN). Inhibition of miR-31 can reduce the transcription factor activity of hypoxia inducible factor 1 (HIF-1) by targeting the biological effects of HIF1AN with the condition of hypoxia. In later hepatic fibrosis could be rescue combining with inhibition of miR-31 and adding heparin-binding EGF-like growth factor (HBEGF).

Keywords
hepatic stellate cells, hypoxia inducible factor 1, hypoxia-inducible factor 1-alpha inhibitor, microRNA-31

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Introduction
Hepatic fibrosis is a main stage in most of chronic liver illnesses, irrespective of etiology, and its progression may cause hepatic cirrhosis or hepatocellular carcinoma. Hepatic fibrosis can be divided into four stages on the basis of METAVIR scoring system (F0, no fibrosis; F1, portal fibrosis lacking septa; F2, portal fibrosis having rare septa; F3, numerous septa lacking cirrhosis; and F4, cirrhosis). Hepatic stellate cells (HSCs) play a major part in hepatic fibrogenesis.1 Mechanisms of pathogenesis plus progression of hepatic fibrosis are complicated. Growing quantity of proof proposes that many microRNAs (miRNAs) critically play part in various developmental phases of hepatic fibrosis, which include activation of HSCs and generation of fibronectin, collagen, and matrix metalloproteinase. Inhibition of miR-31 expression may inhibit the activation of HSCs and reduce the fibrosis-related factors expression. This study provides a promising therapeutic strategy for the treatment of hepatic fibrosis.
of extracellular matrix (ECM) proteins. Carbon tetrachloride is a commonly employed hepatotoxin for in vivo studies in hepatic fibrosis. This is changed to (CCl₄) and (Cl₃OO) by CYP450 in hepar. Moreover, this chemical activates Kupffer cells to generate reactive oxygen species (ROS), like O₂⁻, H₂O₂, and OH, and afterward harm hepar cells. Oxidative stress is the main reason of toxic hepatitis and may result in chronic hepatic disease.

In recent past, massive development has been carried out and it has been recognized that miRNAs play crucial role in regulation of gene expression in many liver disorders, like acute hepatic damage, viral hepatitis, and hepatocellular cancer. It has also been studied of the function of miRNAs in hepatic fibrosis. Forced expression of miR-221/222 enhances to activate HSCs and the prognosis of hepatic fibrosis. Interferon-stimulated miR-195 causes inhibition of HSC proliferation by postponing cell cycle prognosis in G1 to S phase. According to types of miRNAs, miR-31 can be up-regulated to exhibit oncogenic role or down-regulated to play tumor suppressive role in cancers. Notably, miR-31 is overexpressed and oncogenic in colorectal cancer and squamous cell carcinomas (SCCs), but it is down-regulated in serous ovarian cancer. Previous studies showed that miR-31 had expressed miRNA in different way during fibrogenesis, enhancing HSC activation and migration. Inhibition of miR-31-5p leads to decreased glycolysis and adenosine triphosphate (ATP) production, while miR-31-5p overexpression increases them. Hypoxia inducible factor 1 (HIF-1) up-regulates the expression of glycolytic enzymes, and the HIF-1α inhibitor (FIH) inhibits HIF-1 activity. Because FIH is a direct target of miR-31-5p, inhibition of miR-31-5p results in enhanced FIH expression and suppression of HIF-1 signaling, while overexpression of miR-31-5p has the opposite effects. Via this mechanism, miR-31-5p up-regulates aerobic glycolytic genes and maintains energy homeostasis. To further validate the mechanism of miR-31-5p in glycolysis regulation, we show that overexpression or knockdown of FIH rescued the effects of miR-31-5p or miR-31-5p inhibitor on HIF activation and its target gene expression, respectively. Finally, by means of an A549 cell xenograft mouse model, we demonstrate that the miR-31-5p promotes cell proliferation via enhancing glycolysis. HSC activation is a key element in the pathogenesis of liver fibrosis. miR-31 has recently emerged as an important regulator in fibrosis disease. The aim of this study was to determine its role in liver fibrosis. Levels of miR-31 were significantly increased in human primary HSCs during fibrogenesis. In our study, we found miR-31 may contribute to HSC activation and may thereby play an important role in liver fibrosis. In addition, inhibition of miR-31 with antagonist may be benefit for this disorder in the future.

Precious study showed miR-31 suppression nasopharyngeal carcinoma cell growth through targeting hypoxia-inducible factor 1-alpha inhibitor (HIF1AN). miR-31 inhibits the expression of HIF1AN at post-transcriptional levels to enhance effect of hypertrophic scar on the promotion. HIF1AN is a protein which is encoded by HIF1AN gene. HIF1AN inhibits activity of HIF-1 by preventing HIF-1α. Transcription factor HIF-1 has a great role in cellular response to systemic oxygen levels in mammals. HIF-1 has been described to associate with development of hepatic fibrosis, and it plays a critical role in hepatic fibrosis. Despite these reports, the miRNA-based treatment is yet is novel. Human embryonic kidney (HEK) 293 cells are straightforward to grow in culture and to transfect. They have been used as hosts for gene expression. Typically, these experiments involve transfecting in a gene (or combination of genes) of interest, and then analyzing the expressed protein. In current work, we investigated inhibition of miR-31 promoting HIF1AN expression can inhibit the hepatic fibrosis in different stages (F0–F3) induced by CCl₄; however, in F4 stage, down-regulated miR-31 combined with adding heparin-binding EGF-like growth factor (HBEGF) could rescue hepatic fibrosis. This might be a good diagnostic and treatment strategy in the coming era.

**Materials and method**

**Ethical statement**

All animal care and protocols were carried out as per the National Institutes of Health (NIH) guidelines. Also this current work was conducted in accordance with the guidelines of Association for Assessment and Accreditation of Laboratory
Animal Care International. The strategy to handle animals was accepted by Animal Care.

The sample size was taken using the formula of error margin

\[
Z \times \sqrt{\frac{\hat{P}(1-\hat{P})}{n}}
\]

where \(\hat{P}\) is the sample proportion, \(n\) is the sample size, and \(Z\) is the z-score corresponds to our desired confidence levels.

Mice models of hepatic fibrosis

Male BALB/c mice (five mice per group aged 6 weeks) received 200 \(\mu\)L of body weight of carbon tetrachloride \((10\% \text{ CCl}_4)\) mixed with olive oil intraperitoneally, and then initial amount was doubled. To stimulate hepatic fibrosis, \text{CCl}_4 was injected two times a week for 4, 6, 8, and 10 weeks. Same treatment was done with control animals. Serums and tissues hepars from different groups were collected for determination of antioxidant function and quantitative polymerase chain reaction \((\text{qPCR})\). Echo-assisted liver biopsy \((\text{LB})\) was done in all mice by employing modified Menghini needles. For pathological interpretation, LB fragments having eight portal tracts were sufficient. LBs were evaluated in accordance with the META VIR scoring system by expert pathologist blinded to results of liver stiffness \((\text{LS})\) extents. Fibrosis was classified on a 0–4 scale: F0, no fibrosis; F1, portal fibrosis lacking septa; F2, portal fibrosis plus some septa spreading into lobules; F3, many septa spreading to next portal tract or terminal hepatic venules; and F4, cirrhosis.

Finding aspartate transaminase and alanine transaminase activity

Alanine transaminase \((\text{ALT})\) and aspartate transaminase \((\text{AST})\) actions were found with ALT identification kit and AST detection kit \((\text{C009-1 and C009-2, Nanjing Jiancheng Bioengineering Institute, P.R. China})\) through the colorimetric technique. Through Reitman’s method, at 37°C and pH 7.4, ALT acts on substrate composed of alanine and \(\alpha\)-ketone glutaric acid, producing glutamic acid plus pyruvic acid. After half an hour, 2,4-dinitrobenzene hydrazine was added for stoppage of reaction, yielding pyruvate phenyl hydrazone, having reddish brown color in basic environment. Absorbance was read at 505 nm, and then enzyme potential was measured. AST yields glutamic acid plus oxaloacetic acid from \(\alpha\)-ketone glutaric acid plus aspartic acid, creating glutamic acid plus oxaloacetic acid. Oxaloacetic acid converted to pyruvic acid, plus 2,4-dinitrobenzene hydrazine produces 2,4-dinitrobenzene hydrazone, which has reddish brown color in basic environment.

Assay of hyaluronic acid, laminin, and type IV collagen

According to the chemiluminescence method, the instrument AutoLumo A2000 was used to detect serum hyaluronic acid \((\text{HA})\), laminin \((\text{LH})\), and type IV collagen \((\text{C-IV})\), and the testing procedure was completed according to the instruction manual of the kit and the instrument operation procedure.

Hematoxylin and eosin staining

Livers from different groups that treated by \text{CCl}_4 and negative control were instantly fixed in 4\% paraformaldehyde afterward reaction. Microscope slide was used having rehydrated tissue section fixed in alcohol; slide was dipped for half minute by hand in \(\text{H}_2\text{O}\). Slide was dipped into the Coplin jar having Mayer’s hematoxylin and agitates for half minute. Slide was cleaned in \(\text{H}_2\text{O}\) for 60 s. Slide was stained with 1\% eosin \(\text{Y}\) solution for half minute. Sections were dehydrated with two changes of 95\% alcohol plus two changes of 100\% alcohol for half minute. Extract the alcohol with two changes of xylene. 1–2 drops of mounting medium were added, covered by coverslip, and photographed employing the Nikon E80i microscope.

Primary cells plus culture separation

Primary mice HSCs were separated from liver in different times of \text{CCl}_4-treated groups, and cell viability was found by the Trypan Blue exclusion technique. Purity was evaluated by autofluorescence plus immune staining employing anti-desmin antibody. HSCs were cultured in the Opti-MEM medium supplemented with 10\% fetal bovine serum \((\text{FBS})\). HEK 293T cells were got from the American Type Culture Collection. 293T cells were cultured in the Opti-MEM medium. Whole cells were kept at 37°C with 5\% carbon dioxide.
For detection of expression of miRNAs, 2 μg of total RNA was employed to prepare cDNA by reverse transcription through the TIANScript RT kit. Expression values became normal to control endogenous small RNA U6. Bulge-Loop miRNA qPCR Primer Set was employed to find expression of miRNAs by the real-time quantitative polymerase chain reaction (qRT-PCR) technique having the SYBR Green qPCR Master Mix (Tiangen Biotech, Beijing, PR China). Total RNA was isolated, reverse transcribed by the PrimeScript™ RT Reagent Kit (TaKaRa Biomedical Technology, Beijing, PR China). Primers for miR-31, fibronectin (FN), Col 1α1, and Col 3α1 were made employing the Primer 5.0. Primers of GAPDH and β-actin (Table 1) were employed as internal standard to normalize expression plus identification of variations. Reaction mixtures were made following the SYBR green kit principle plus qRT-reverse transcription PCR was done by the 7900HT PCR instrument. Data became normal to four internal control genes, and relative expression levels were found by the 2−ΔΔCt technique. For assessment of data, software employs mean value of Ct value of selected four internal control genes for making baseline correction. Data of mRNA expression were assessed by the GeneCopoeia-FulenGen qPCR Array data analysis system online.

### Real-time qPCR for miRNA and mRNA expression

For detection of expression of miRNAs, 2 μg of total RNA was employed to prepare cDNA by reverse transcription through the TIANScript RT kit. Expression values became normal to control endogenous small RNA U6. Bulge-Loop miRNA qPCR Primer Set was employed to find expression of miRNAs by the real-time quantitative polymerase chain reaction (qRT-PCR) technique having the SYBR Green qPCR Master Mix (Tiangen Biotech, Beijing, PR China). Total RNA was isolated, reverse transcribed by the PrimeScript™ RT Reagent Kit (TaKaRa Biomedical Technology, Beijing, PR China). Primers for miR-31, fibronectin (FN), Col 1α1, and Col 3α1 were made employing the Primer 5.0. Primers of GAPDH and β-actin (Table 1) were employed as internal standard to normalize expression plus identification of variations. Reaction mixtures were made following the SYBR green kit principle plus qRT-reverse transcription PCR was done by the 7900HT PCR instrument. Data became normal to four internal control genes, and relative expression levels were found by the 2−ΔΔCt technique. For assessment of data, software employs mean value of Ct value of selected four internal control genes for making baseline correction. Data of mRNA expression were assessed by the GeneCopoeia-FulenGen qPCR Array data analysis system online.

### RNA-seq techniques

Total RNA from liver was isolated from F0 to F4. Samples of total RNA (1 mg/mL) with RNA Integrity Numbers in the range 7.4–9.4 were employed. PolyA+ fraction was cleansed plus fragmented, changed to double stranded cDNA and treated through following enzymatic treatment of end-repair, dA-Tailing, and ligation to adapters, by Illumina’s “TruSeq Stranded mRNA Sample Preparation Part # 15031047 Rev. D” procedure. Adapter-ligated library was done through PCR having Illumina PE primers, cDNA was purified in this way and applied to an Illumina flow cell for cluster generation and sequenced on an Illumina HiSeq2000, employing standard procedure RNA-seq gene list preranked by statistics was employed, setting “gene set” as permutation technique, and it was ran with 1000 permutations. Only those gene sets with significant enrichment levels (false discovery rate (FDR) q-value) were taken into account.

### Western blot analysis

Proteins separated from various phases were shifted to nitrocellulose membranes afterward sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Incubation of membrane was done with 5% skimmed milk powder, later with the collected polyclonal anti-FN antibody (1:800 dilution), anti-Col 1α1 antibody (1:2000), and anti-Col 3α1 (1:1500) (Abcam, Eugene, OR, USA). We used GAPDH as a housekeeping gene. Then, incubation of blots was done with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (1:2000 dilutions, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Anti-tubulin was employed as internal standard. Enhanced ECL chemiluminescence reagent kit was employed to

### Table 1. Primers employed in qRT-PCR.

| Gene name | Primer sequence (5'→3') | Tm | Product (bp) |
|-----------|--------------------------|----|--------------|
| GAPDH     | Forward: AGGTCCGCTGTAACGGAAGATTTG | 58 | 95 |
|           | Reverse: GGCTGTGATGCGAACA | 56 |
| β-actin   | Forward: GCTACAGCTCTTACACACCAC | 57 | 134 |
|           | Reverse: CTATATGCTCTCTCAGAGGA | 56 |
| Col 1α1   | Forward: TGACTGGAAGAGCCCCAGAGAT | 59 | 151 |
|           | Reverse: GTTCGGCTAGTGTAACAGGT | 58 |
| Col 3α1   | Forward: TGATGGAAAAACCCAGGACCTC | 55 | 203 |
|           | Reverse: CAGCTTCTCCCCATCTTTACA | 55 |
| miR-31    | Forward: TCTTTCTACCATGGCAGTC | 56 | 219 |
|           | Reverse: TGAGAGCTTGCAACGTCAG | 56 |
| HIF1α     | Forward: TGCCAGTTTTTGAGCATAG | 55 | 198 |
|           | Reverse: GCACATGCTAGTCAGTTCGAT | 57 |
| HIF-1α    | Forward: TGTCCTCATGACCAGATCA | 55 | 169 |
|           | Reverse: TCTCTGCTGTGTAACATTC | 56 |
| FN        | Forward: CTTTGTGATTGTTAGGACCT | 56 | 221 |
|           | Reverse: ACCAGAAAACCTTGGCAATGG | 55 |
detect immunoreactive bands. Target bands were quantified by the ImageQuant TL 8.1. For densitometric assessment, the ImageJ software was employed.

**Inhibitor interference**

The miR-31 inhibitor (TuoRan biological, China) or ddH₂O as a control was added to conditioned medium for 48 h. By adding miR-31 inhibitor to primary mice HSCs of F1–F4 to analyze the effects on miR-31, HIF1AN and HIF-1 mRNA expression levels, and F0 (4–10 weeks) as a control. The 50 μg miR-31 inhibitor was dissolved in 100 μL water ddH₂O.

Mice heparin-binding EGF-like growth factor (mHBEGF) was purchased from the Amyjet Scientific. By adding mHBEGF and miR-31 inhibitor to primary mice HSCs of F4 to analyze the co-treatment of inhibition of miR-31 with mHBEGF. The concentration of mHBEGF was diluted to 0.1 ng/mL.

**Statistical analysis**

Data were analyzed by the GraphPad Prism 5.1 (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) was employed to assess data. Results were showed as mean values ± standard error of the mean (SEM) of various groups. Result was summarized and tabulated in Microsoft Excel file. Statistical analysis was carried out employing the MedCalc program and WINK Statistical Data Analysis Research Software. LS values and scores were showed as mean values and standard deviation (SD). Spearman’s rank correlation coefficient was employed to analyze correlations between histological findings and several predictive scores of hepatic fibrosis. Two-way ANOVA was employed to relate mean values for various phases of fibrosis in novel scoring system. Multiple regressions were used to find new hepatic fibrosis scores. Diagnostic performance of novel scoring system was analyzed employing receiver operating characteristic (ROC) designed for identification of fibrosis: (F ≥ 1, META VIR score), significant fibrosis (F ≥ 2), severe fibrosis (F ≥ 3), and cirrhosis (F = 4). Optimal cut-off values were selected so that sensitivity and specificity were highest. Sensitivity and specificity were measured employing standard protocol. Moreover, 95% confidence intervals were measured for every predictive test and were used to relate area under the receiver operating characteristic (AUROC) curves.

**Gene array analysis**

This method includes preparing labeled cDNA from two or more sources, hybridizing them to alike gene arrays. The process is performed in molecular biology laboratories having necessary facilities.

This method is a good technique in molecular biology these days and will become a useful tool in molecular medicine in future. Large number of illnesses would define in a “customized” fashion in accordance with array of genes in the specific people having diseases.

**Results**

**RNA sequence analysis**

We use RNA-seq reading mapped fold induction of miRNA in F0–F4 stages of hepatic fibrosis, and we identified nine of these miRNAs performed significant difference in all the four stages (Figure 1). Among them, miR-31 has shown a specific expression that it altered much more than other miRNAs in F3 stages (Figure 2(a)). We decide to search for the pathway that associated with difference expression miRNA target genes. Based on KEGG, MAPK signaling pathway and focal adhesion have shown the most significant enrichment (Table S1).
regeneration, connective tissue hyperplasia and fibrous septum development, hepatic lobule structural destruction, and pseudolobular formation had shown a rising state by times.

**miR-31 is mostly up-regulated in HSCs in hepatic fibrosis**

Histopathological examination had shown that CCl₄-induced mice for 4–10 weeks could present the four stages of hepatic fibrosis. Primary-activated HSCs and hepatocytes were separated from CCL₄-stimulated hepatic fibrosis BALB/c mice model. We used miRNA gene array analysis to explore which miRNAs involved in the regulation of hepatic fibrosis formation mechanism, and the results of miR-31 showed the highest expression. Relative expression level of miR-31 in F0 tissues was chiefly different in F1–F4 (Figure 2(b)). With the time of CCl₄ induction, the degree of fibrosis increased, and the expression levels of miR-31 had shown an upward trend from F1 to F4 stages. Expression of miR-31 was also enhanced profoundly at F1–F4 groups during HSC activation; the trend was the same as the result in hepatic tissues. In the four stages of CCl₄-induced HSCs, three fibrosis markers—FN, Col1α1, and Col3α1—were also significantly increased in CCl₄-induced model mice of F1–F4 (Figure 2(c) and Table S2). Unexpectedly, the model was successfully established. These findings suggest that miR-31 was enhanced in activated HSCs throughout hepatic fibrosis.

**Suppression of HSC activation by inhibiting miR-31**

To investigate roles of miR-31 in detail, the effect of miR-31 inhibition was tested from F1 to F4
Figure 2. (a) Histopathological examination. The pathology slices of hepatic tissues of BALB/c mice of F0–F4 by microscope (200×, HE). F1–F4 groups were mice treated with CCl4 for 4–10 weeks, and F0 treated with olive oil as a control. (b) Effects of CCl4-induced HSC fibrosis index (A–D). Relative expression of fibrosis markers (FN, Col 1α1, and Col 3α1) among different groups. F0 represented mice treated with olive oil; F1 represented mice treated with CCl4 for 4 weeks; F2 represented mice treated with CCl4 for 6 weeks; F3 represented mice treated with CCl4 for 8 weeks; F4 represented mice treated with CCl4 for 10 weeks. ****P<0.0001. (c) Effects of inhibition of miR-31 on fibrosis marker mRNA expression levels (A–D). Inhibition of miR-31 can decrease FN, Col 1α1, and Col 3α1 mRNA expression levels in HSCs from F1 to F3 stages, and no significant difference in F4 stage. Inter-NC represented hepatocytes isolated from CCl4-induced mice; inter-miR-31 represented hepatocytes isolated from CCl4-induced mice treated with miR-31 inhibitor.
stages. Findings showed that miR-31 inhibition was meaningfully reduced FN, Col 1α1, and Col 3α1 mRNA expression levels from F1 to F3 stages, whereas no significant difference in F4 stage (Figure 3(a)). Similar results were presented in FN, Col 1α1, and Col 3α1 protein expression levels (Figure 3(b)). The findings revealed that miR-31 inhibition meaningfully inhibited HSC activation from F1 to F3 stages but no F4 stages.

miR-31 regulates HIF activation on HSCs by targeting HIF1AN under hypoxia condition

Under hypoxia condition, we found that inhibition of miR-31 from F1 to F4 inhibits the fibrosis process, and HIF1AN expression was meaningfully increasing throughout HSC inactivation, which showed entirely contradictory tendency to miR-31. With the improvement of the fibrosis process, HIF-1 mRNA expressions were decreasing (Figure 3(c)) from F1 to F3 stages. In HSC activation environment, HIF-1 proteins were relative increasing (Figure 3(d)).

Combining inhibition of miR-31 with HBEGF rescue late stage fibrosis process

HBEGF has a significant part in wound healing, cardiac hypertrophy, cardiac development, and role. Initially recognized in conditioned media of human macrophage-like cells, HBEGF is an 87-amino acid protein that shows greatly regulated gene expression. Ectodomain shedding effects in soluble mature kind of HBEGF that affects mitogenicity plus chemotactic factors for smooth muscle cells also for fibroblasts. We can see in Figure 3(d) that inhibition of miR-31 expression can rescue hepar fibrosis in the F0–F3 stages, but not in the F4 stage. It reported that endogenous and exogenous HBEGF inhibited HSC activation in primary culture, and HBEGF enhanced HSC migration. We hypothesized that whether we could combine miR-31 with HBEGF to deal with HSCs in the F4 stage. In the case of adding HBEGF and inhibiting miR-31, the mRNA expressions of FN, Col 1α1, and Col 3α1 have no significantly difference compared to those in F0 stage.

Discussion

Combined with observation of pathological sections, we used CCl4 to induce the hepatic fibrosis four stages (F1–F4). Hepatic fibrosis is reversible growth of extracellular matrix which is developed because of chronic damage in which nodules may not so far synthesized. LH is a non-collagen glycoprotein in the extracellular matrix, mainly in the cell basement membrane transparent layer, and C-IV to maintain the basement membrane of the grid structure. HA is the main component of proliferating cells and migrating cells extracellular matrix, especially in embryonic tissue. Because of the characteristics of hyaluronic acid, cells can be separated from each other, allowing cells to move easily and proliferate and prevent cell differentiation. With the inflammation of the hepatic disease, fibrous tissue formation process, a large number of collagen deposition and a variety of collagen has increased. Among these the most important is the basement membrane of C-IV. These three factors could reflect the amount of fiber generated within the hepatic and its damage.

Compared to study that miR-31 expression was not changed in the whole liver, but by microarray profiling and RT-PCR, it was observed that miR-31 expression in HSCs from rats, mice plus humans

Table 2. Effects of hepatic function indexes plus fibrosis-related factors induced by CCl4 (4–8 weeks) or olive oil in BALB/c mice.

| Week | Group | F0* | F1 | F0 | F2 | F0 | F3 | F0 | F4 |
|------|-------|-----|----|----|----|----|----|----|----|
|       | AST (U/g protein) | 112.62 | 209.32*** | 128.71 | 421.42**** | 116.73 | 543.12**** | 135.25 | 682.42**** |
|       | ALT (U/g protein) | 45.31 | 182.51**** | 54.21 | 264.32**** | 62.31 | 421.12**** | 50.43 | 573.43**** |
|       | C-IV (ng/mL) | 54.13 | 88.32**** | 56.34 | 95.32**** | 63.16 | 115.23**** | 59.81 | 142.84**** |
|       | LH (ng/mL) | 63.23 | 78.21**** | 64.72 | 93.42**** | 69.65 | 110.53**** | 70.43 | 129.32**** |
|       | HA (ng/mL) | 63.21 | 183.42**** | 67.21 | 214.42**** | 82.31 | 378.32**** | 69.16 | 483.21**** |

SEM: standard error of the mean; AST: aspartate transaminase; ALT: alanine transaminase; C-IV: type IV collagen; LH: laminin; HA: hyaluronic acid.

*F0: BALB/c mice treated with olive oil; F1–F4: BALB/c mice treated with CCl4 for 4–10 weeks.

Values of significant differences among the F0 and F1–F4 at the same week (**P < 0.001 and ****P < 0.0001).

Pooled SD (five BALB/c mice per each treatment).
was meaningfully enhanced throughout HSC stimulation in culture. Generally, miR-31 expression levels were unaffected in total liver RNA extracts from fibrotic rat plus human samples. However, it was revealed that miR-31 was mainly up-regulated in HSCs, not in hepatocytes throughout fibrogenesis. Therefore, it is concluded that miR-31 may stimulate liver fibrosis.

To some extent, we supposed that HSC behavior was probably regulated by miR-31. We noted that miR-31 is an essential regulator of the pathological activities of lung fibroblasts. However, we urgently explore the biological function of miR-31 in different stages of hepatic fibrosis. FN is one of the major cell adhesion molecules; changes in FN expression, degradation, and combination are closely related to great cases of pathologies, like cancer and fibrosis. Col 1α1 and Col 3α1 have a hemostatic function. When the collagen synthesis and deposition is greater than the degradation and absorption, the increase in intrahepatic collagen fibers was gradually fibrosis. When miR-31 was inhibited, Col 1α1, Col 3α1, and FN significantly decreased from F1 to F3 stages, but no significant change in F4 stage. We hypothesized that inhibition of miR-31 was able to reverse the fibrosis of certain periods. When fibrosis developed to cirrhosis, substitution of miR-31 only could not reduce fibrosis.

Figure 3. (a) Effects of inhibition of miR-31 on fibrosis marker protein expression levels. The protein levels of FN, Col 1α1, and Col 3α1 were decreased by inhibition of miR-31 from F1 to F3 stages, no significant difference in F4 stage, and assessed by the western blot. (b) Effects of inhibition of miR-31 on fibrosis marker mRNA expression levels (A–D). Inhibition of miR-31 can decrease FN, Col 1α1, and Col 3α1 mRNA expression levels in HSCs from F1 to F3 stages, and no significant difference in F4 stage. Inter-NC represented hepatocytes isolated from CCl4-induced mice; inter-miR-31 represented hepatocytes isolated from CCl4-induced mice treated with miR-31 inhibitor. (c) Effects of inhibition of miR-31 on HIF1AN and HIF-1α protein expression levels (A–D). Inhibition of miR-31 can decrease miR-31 and HIF-1 protein expression levels whereas increase HIF1AN protein expression levels in HSCs from F1 to F3 stages with the condition of hypoxia. Inter-NC represented hepatocytes isolated from CCl4-induced mice; inter-miR-31 represented hepatocytes isolated from CCl4-induced mice treated with miR-31 inhibitor. (d) Combining inhibition of miR-31 with adding HBEGF rescue late hepatic fibrosis. Relative mRNA expressions of fibrosis markers by combining inhibition of miR-31 with adding HBEGF. Relative protein expressions of fibrosis markers by combining inhibition of miR-31 with adding HBEGF.
miR-140, and miR-193) and nine down-regulated (miR-341, miR-20b-3p, miR-15b, miR-16, miR-375, miR-122, miR-146a, miR-92b, and miR-126) miRNAs.

By TargetScan, miRDB, PicTar, miRnada, and miRwalk algorithms, HIF1AN was supposed to be putative target of miR-31.22 We validated HIF1AN being negatively regulated by miR-31 among all the fibrosis process (F1–F4). At the same time, FIH mRNA and protein expression levels were significantly higher in fibrosis process than normal. In hypoxic conditions, Col 1α1, Col 3α1, and FIH protein expression levels had reduced by times. We suggest that the inhibition of miR-31 significantly decreased HSC activation, as a result of HIF1AN increased. We had known HIF1AN could interact with HIF-1. Thus, we suggested that miR-31 could alter the transcription factor activity of HIF-1 by targeting the biological action of HIF1AN in F1–F3 stages. Inhibition of miR-203 may rescue suppressed PI3K expression and stem cell action. JNK and c-Jun to continue to HBEGF–EGFR signaling mechanism.

Despite the current achievements, to cure hepatitis, medical approaches to enhance reverse of hepatic fibrosis remain inadequate. miRNA-based treatment methods might signify novel alternate to recent approaches in management of hepatic diseases. Founded on function of an miRNA candidate and its expression in unhealthy tissue. Many methods may hinder miRNAs revealing promoting the function in fibrotic tissue.23 We found that inhibition of miR-31 could rescue F1–F3 stage hepar fibrosis, but no in F4. Previous study demonstrated that gene expression of HBEGF had a positive significant correlation with that of collagen in human fibrotic livers.24 So, we combined inhibition of miR-31 and added HBEGF, and it showed that co-treating can rescue the F4 stage hepar fibrosis.

**Conclusion**

In general, our studies identified that the inhibition of miR-31 expression promotes the expression of target gene HIF1AN and inhibits CCl₄-induced hepatic fibrosis, and combined with adding HBEGF could rescue late hepatic fibrosis. It might be a promising diagnostic plus treatment strategy in coming days.

Limitation of the study: miRNAs play a critical role in hepatic fibrogenesis, and their promising use as treatment and biomarkers are very interesting. Although, standardization is required in investigation procedures and, moreover, their role and mode of activity is required to further investigated for translating them into clinical settings.

**Declaration of conflicting interests**

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**Supplemental material**

Supplemental material for this article is available online.

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