miR-98-5p as a Novel Biomarker Suppress Liver Fibrosis by Targeting TGFβ Receptor 1

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Research Article

Keywords: Liver Fibrosis, miRNAs, HBV Infection, Biomarker, miR-98-5p, TGFβ R1, Bioinformatic Analysis, Differential Expression, Target Genes, Signaling Pathway

Posted Date: August 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-520986/v2

Version of Record: A version of this preprint was published at Hepatology International on February 21st, 2022. See the published version at https://doi.org/10.1007/s12072-021-10277-5.

miR-98-5p as a novel biomarker suppress liver fibrosis by targeting TGFβ receptor 1

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Keywords: Liver Fibrosis; miRNAs; HBV Infection; Biomarker; miR-98-5p; TGF β R1; Bioinformatic Analysis; Differential Expression; Target Genes; Signaling Pathway

Abstract

Hepatic fibrosis is the repair reaction of excessive deposition and abnormal

distribution of extracellular matrix after various liver injuries, especially chronic HBV infection, which is a key step in the development of various chronic liver diseases to cirrhosis. Recent studies show that microRNAs (miRNAs) can regulate a series of liver fibrosis-related gene express and play an important role in the development of liver fibrosis. To detect the miRNAs expression profiling and to screen the differentially expressed miRNAs in patients with HBV-related liver fibrosis, the whole blood was collected from the HBV-related liver fibrosis patients (S2/3, n=8) based on Scheuer's staging criteria. In addition, healthy volunteers(n=7) served as the control group. The expression of plasma miRNAs was detected by IlluminaHiSeq sequencing. Cluster analysis and target genes prediction of differentially expressed miRNAs were performed. Gene ontology (GO) enrichment analysis and KEGG pathway enrichment analysis of differentially expressed miRNAs target genes were performed. Compared with the healthy control group 77 miRNAs were screened out from the liver fibrosis group, among which 51 miRNAs were up-regulated and 26 miRNAs were down-regulated. Pathway annotations for the target genes of the miRNAs identified were found that it participated in many signal pathways including MAPK signaling pathway, TNF signaling pathway, Notch signaling pathway, phosphatidylinositol signal system and so on. According to the bioinformatic analysis，miR-98-5p were selected for function research among the differentially expressed miRNAs.MiR-98-5p prevents liver fibrosis by targeting TGF β R1 and blocking TGF β 1/Smad3 signaling pathway. In addition, serum miR-98-5p levels were measured from a total of 70 recruited patients with chronic HBV infection and 29 healthy individuals as controls. We found that serum miR-98-5p level was significantly lower in patients with live fibrosis than in healthy controls and HBV carriers ($P<0.05$). Those results suggest that miR-98-5p could be a potential therapeutic target for liver fibrosis.

Introduction

The prevalence of HBV infection is worldwide, but the epidemic status varies greatly in different regions. According to WHO, the more 257 million people are infected

with chronic HBV, the more 887,000 people die of HBV-related diseases worldwide every year, among which cirrhosis and primary hepatocellular carcinoma account for 52% and 38% respectively(1). According to statistics, the prevalence rate of HBsAg in the general population of our country is about 5%-6% at present, and chronic HBV infection is about 7000 cases, among which chronic HBV patients are about 20 million to 30 million cases(2).Liver fibrosis is a pathological repair response to chronic injury and an important link in the development of various chronic liver diseases to cirrhosis. Activation of hepatic stellate cells (HSCs) plays an important role in the process of hepatic fibrosis(3). At present, the methods of early diagnosis of liver fibrosis, includes liver biopsy, imaging or laboratory examination, are not satisfactory. So reliable non-invasive biomarkers are required for early diagnosis of liver fibrosis.

Recent studies show that miRNAs can regulate a series of liver fibrosis-related gene express and play a very important role in the development of hepatic fibrosis. MiRNAs are a class of endogenous non-coding single stranded RNAs of~22 nucleotides in length, which are stable in peripheral blood(4).They can induce mRNAs degradation or inhibit its translation by pairing with the 3'untranslated region (3'UTR) of mRNAs, thus exerting its negative regulation at the post-transcriptional level(5). MiRNAs play an important role in cell differentiation, biological development and the development of diseases(6). Plenty of evidence has showed that miRNA plays an important role in the process of liver fibrosis and may be used as an indicator to monitor liver fibrosis (5; 7). However, there has no record of a systemic screening for liver fibrosis associated miRNAs in patients infected with HBV. In the present study, plasma miRNA biomarkers associated with fibrosis in patients with chronic HBV infection were screened by IlluminaHiSeq sequencing. We detected the microRNA expression profiles in patients with HBV-related liver fibrosis and healthy controls and screened out the differentially expressed miRNAs. Subsequently, cluster analysis and target gene prediction were performed for the differentially expressed miRNAs. Gene ontology (GO) analysis and KEGG pathway enrichment analysis also were performed on the differentially expressed target miRNA genes. MiR-98-5p was

selected according to bioinformatics and was discussed its mechanism in the progress of hepatic fibrosis. Currently, we found that miR-98-5p confirmed to be significantly reduced during the progress of liver fibrosis, and over-expression of miR-98-5p analogues in the LX2 cells, could reduce the TGFβR1 expression, and suppress the TGFβ/Smad signaling pathway related genes. Finally, we further determined serum miR-98-5p levels and found that they were notably decreased in patients with liver fibrosis, compared with HBV carrier and healthy controls. So, based on the potential correlation between miR-98-5p and liver fibrosis, we hypothesized that serum miR-98-5p may be a novel biomarker for liver fibrosis.

Results

Patients' information

All patients were strictly enrolled according to the inclusion and exclusion criteria. Fibrosis score of liver biopsy in liver fibrosis was based on Scheuer's staging criteria (S2 indicated HBV carrier; S3 indicated liver fibrosis; S4 indicated liver cirrhosis; S5 indicated hepatic carcinoma). The detailed clinical data of the two groups was presented in Table 1. There was no significant difference in age and sex between the liver fibrosis group and the healthy group. The ALT and AST levels of the liver fibrosis group were significantly higher than that of the health group. The HBV-DNA average level was 2.47E+5 IU/ml and HBeAg positive rate was 50% in the liver fibrosis group.

Microarray screening for microRNAs associated with liver fibrosis infected with HBV

The transcription starting sites of miRNA are mostly located in gene spacers, introns and reverse complementary sequences of coding sequences. The precursors of miRNA have a symbolic hairpin structure, and the formation of mature body is realized by the splicing of Dicer/ Dclase. According to the biological characteristics of miRNAs, the software miDeep2 was used to identify known and new miRNAs when the sequence was aligned to the reference genome (10). Microarray data revealed that a total of 1,973 miRNAs were predicted from all samples, of which 1,389 were

known and 584 were newly discovered. Next, using |log2(FC)|≥1 and FDR≤0.01 as selection criteria, differentially expressed miRNAs were analyzed. Compared with the healthy control group, 77 miRNAs were screened out from the liver fibrosis group, among which 51 miRNAs were up-regulated and 26 miRNAs were down-regulated **(Fig.1A)**. The results of cluster analysis and target gene prediction showed that the target genes of differentially expressed miRNAs were mainly involved in biological processes, such as cell growth, maintenance and signal transduction, etc. Involved in cell components, such as the proteasome, cell membrane and extracellular matrix, etc. Involving molecular functions, such as catalytic activity, transport activity and binding activity, etc. **(Fig.1B)**. Pathway annotations for the target genes of the miRNAs identified were found that it participated in many signals including MAPK signaling pathway, TNF signaling pathway, Notch signaling pathway, phosphatidylinositol signal system and so on **(Fig.1C-D)**.

The expression level of miR-98-5p is reduced in the liver fibrosis group

The biological processes involved in the target genes corresponding to the differentially expressed RNA were analyzed, and it was found that the target genes of 26 down-regulated miRNA were all related to Collagen type IV**(Fig.2A)**. It has been reported that in the process of human intervertebral disc degeneration, miR-98-5p can target the IL-6/STAT3 signaling pathway to promote the degradation of extracellular matrix(11). In myocarditis, miR-98-5p plays a role by targeting Fas/FasL, and overexpression of miR-98-5p can inhibit the apoptosis of cardiomyocytes, thus affecting the pathological process of myocarditis (12). In non-small cell lung cancer, miR-98-5p can target PAK1, inhibit its translation, and inhibit the proliferation and invasion of cancer cells(13) . In addition, it has been reported that miR-98-5p protects endothelial cells against apoptosis caused by hypoxia/reoxygenation by targeting caspase-3 (14). In glial cells, overexpression of miR-98-5p can inhibit cell invasion by down-regulating IKKε (9). However, the role of miR-98-5p in liver fibrosis has not been reported. Compared with the healthy group, the expression of miR-98-5p in the peripheral blood of liver fibrosis group was significantly lower **(Fig.2B, ***P<0.001).**

miR-98-5p inhibits migration and proliferation of LX2 cells

To explore whether miR-98-5p are involved in regulating migration and proliferation of LX2 cells, we used CCK8, wound healing and migration methods to detect cell migration, invasion and proliferation. Over-expression miR-98-5p mimic in LX2 cells resulted in significantly suppressing the wound healing and migration, compared with NC mimic group **(Fig.3A)**. miR-98-5p inhibitor led to acceleration of the wound healing and migration in LX2 cells compared with NC inhibitor group **(Fig.3B)**. Over-expression miR-98-5p mimic in LX2 cells resulted in significantly suppressing LX2 cells proliferation in 24h, 48h and 72h **(Fig.3C, *P<0.05, **P<0.01, ***P<0.001)**, while miR-98-5p inhibitor led to increasing proliferation of LX2 cells **(Fig.3D, *P<0.05, **P<0.01, ***P<0.001)**. These findings suggested that miR-98-5p negative regulates migration, invasion and proliferation of LX2 cells. Taken together, these results demonstrated that miR-98-5p reduced HSCs activation in vitro.

miR-98-5p suppress the expression of TGFβR1

TGFβ1/Smad3 signaling pathway plays an important role in the process of liver fibrosis(15). The signal transduction process of TGFβ1/Smad3 inducing HSCs activation is as follows: First, TGFβ1 binds to TGFβ type II and type I receptors to form a receptor heteromerer complex, which simultaneously activates the phosphorylated kinase of the type II receptor to phosphorylate the type I receptor, and the phosphorylated type I receptor further phosphorylates Smad3 or Smad2 in the cytoplasm through signal amplification (16). Activated Smads will transfer the signal of ligand and receptor interaction from the cell membrane and cytoplasm to the nucleus, and then cooperate with other nuclear factors to activate or inhibit the transcription of target genes (17-19). Therefore, inhibition of TGFβ1/Smad3 signaling pathway can inhibit the formation of fibrosis and may be a potential and effective anti-fibrosis treatment strategy. Bioinformatics predicted that miR-98-5p could target TGFβR1 and block the TGFβ1/Smad3 signaling pathway. To explore whether miR-98-5p are involved in regulating TGFβRI expression, we integrated the results of the prediction software programs TargetScan and PicTar. MiR-98-5p predicted to target TGFβRI, were found by all two programs. Our previous results showed that miR-98-5p was down-regulated in liver fibrosis. Further research found that

over-expression miR-98-5p mimic in LX2 cells resulted in significantly suppress the expression of TGFβR1 in both mRNA and protein level. At the same time, compared with the control group, α-SMA, coll1A1, coll3A1, coll5A1 coll1A1 and Smad3 were significantly reduced in mRNA level **(Fig.4A)**. The proteins of α-SMA, collagen Ⅰ, collagen Ⅲ, p-Smad3 were significantly reduced when LX2 cells were transfected with miR-98-5p, which suggested that miR-98-5p inhibited the HSCs activation. To further explore the mechanism underlying miR-98-5p regulation of HSCs activation, we assessed the effect of miR-98-5p on the TGF-β1/Smad3 signaling pathway, which plays an important role in HSC activation and collagen synthesis. First, miR-98-5p mimic and mimic control were transfected into LX2 cells, respectively. After 24 hours the cells were incubated in the presence of TGF-β1 (5ng/mL) for another 24 hours. Interestingly, miR-98-5p over-expression caused decreased α-SMA, collagen Ⅰ, collagen Ⅲ, p-Smad3 **(Fig.4B)**. These findings suggested that miR-98-5p negative regulates the TGF-β1/Smad3 signaling pathway. Taken together, these results demonstrated that miR-98-5p reduced HSC activation and ECM production *in vitro*.

MiR-98-5p directly targets TGFβR1

According to bioinformatics analysis, there are two miR-98-5p action sites on TGFβR1 3'UTR. Previous experimental results also suggested that miR-98-5p may play a role in fibrosis by binding to TGFβR1 3'UTR. MiR-98-5p suppress the expression of TGFβR1 in both mRNA and protein level **(Fig.5A)**. And miR-98-5p has potential binding sequences in the 3'UTRs of TGFβR1**(Fig.5B)**. Whether or not TGFβR1 are miR-98-5p target genes, we construct the pmirGLO-luciferase reporter containing either the wild-type (WT) or mutated (MUT) miR-98-5p binding sequences in the 3'UTRs. Treatment with miR-98-5p mimic significantly reduced the activity of firefly luciferase with the wild-type second binding site but not mutant 3'-UTR of TGFβR1 **(Fig.5B-C)**. These results strongly indicate that TGFβR1 are direct targets of miR-98-5p.

Mir-98-5p could be a potential biomarker for liver fibrosis in patients with chronic HBV infection

The serum levels of miR-98-5p in patients with liver fibrosis were significantly lower

than those in healthy controls and HBV carriers **(Fig.6, *P<0.05)**. The detailed clinical data of the two groups was presented in Table 2. There was no significant difference in age and sex between the HBV infection group and the healthy group. The HBV-DNA average level was 2.83E+5 IU/ml and HBeAg positive rate was 69.77% and the HBsAg average level was 3.25 IU/ml in the liver fibrosis group. Interestingly, the level of miR-98-5p was no difference between healthy controls and HBV carriers. These results partially suggest that serum miR-98-5p could be a potential biomarker for liver fibrosis in patients with chronic HBV infection.

Discussion

Most chronic liver diseases will present with liver fibrosis(3). Further development of liver fibrosis can cause cirrhosis, manifested as liver dysfunction and portal hypertension. Hepatic fibrosis is histologically reversible, and cirrhosis is difficult to reverse(20).Therefore, the early diagnosis of liver fibrosis becomes especially important. However, liver biopsy is still the gold standard for the diagnosis of liver fibrosis, it has some limitations because of its invasive(21). Many studies tried developing non-invasive tests to substitute liver biopsy for fibrosis assessment(22). However, the noninvasive diagnosis and effective intervention of liver fibrosis are still unsatisfactory. In recent years, miRNAs have become a hot topic in biology research, which plays an important role in the process of liver fibrosis.

MiRNAs are involved in cell differentiation, biological development, and the progression of various diseases. Different diseases have different miRNAs expression profiles, suggesting that miRNAs can be used as an effective non-invasive diagnostic marker(5). MiRNA may play an important role in the progress of liver fibrosis(23). Maubach, G. et al used microarrays to detect the differential expression of miRNAs during the activation of HSCs and found that 16 miRNAs were up-regulated and 26 were significantly down-regulated in HSCs activated for 10 days in vitro, compared with HSCs in the static state(24). Guo et al found that12 miRNAs were upregulated, and 9 miRNAs were downregulated in activated HSCs compared with rat quiescent HSCs(25). Our study found that compared with the healthy control group, 77

miRNAs were screened out from the liver fibrosis group, among which 51 miRNAs were up-regulated and 26 were down-regulated. The target genes of differentially expressed miRNAs were mainly involved in biological processes, such as biological adhesion, cell aggregation and locomotion, etc. They also participate in molecular functions, such as translation regulator activity, channel regulator activity and electron carrier activity, etc. Pathway annotations for the target genes of the miRNAs identified were found that it participated in many signal pathways including MAPK signaling pathway, TNF signaling pathway, Notch signaling pathway, phosphatidylinositol signal system and so on. Among the above miRNAs screened by us, some miRNAs have been reported in relevant studies. For example, Guo et al. found that in the activation process of hepatic stellate cells, miR-16-5p may inhibit the anti-apoptotic effect by targeting Bcl2 and caspase signaling pathways(25). During TGF-β1-induced activation of hepatic stellate cells, the expression of miR-146a-5p is decreased, and miR-146a-5p regulates TGFβ-induced differentiation of HSC by targeting Smad4(26). The expression level of miR-335-3p was down-regulated during the activation of HSCs, and the overexpression of miR-335 could significantly inhibit the activation and migration of HSCs (27). In addition, miR-126 can bind to the 3'UTR of nuclear factor kappa B inhibitor α (IF-κBα), inhibit the expression of IκBα and increase the expression of NF-κB protein. On the contrary, miR-126 knockout inhibited the activity of NF-κB by upregulating the expression of IκBα(28). In addition, it has been reported that miR-483-5p, miR-483-3p, miR-122-5p and miR-193 are involved in the occurrence and development of liver fibrosis(29-31). Some miRNAs screened out have not been reported to be involved in the process of liver fibrosis, but there are literature suggesting that they play an important role in the process of fibrosis of other organs, such as miR-192-5p involved in the process of cardiac fibrosis(32). MiR-199a-5p plays a role in pulmonary fibrosis (33); MiR-26a is down-regulated in the lung tissues of Idiopathic pulmonary fibrosis (IPF) patients and reverses pulmonary fibrosis (34). However, miR-3651, miR-4467, miR-365a-5p and miR-548J-5p have not been reported to be involved in fibrosis. Unfortunately, miRNA as a non-invasive diagnosis and effective intervention for liver fibrosis is still not

satisfactory.

There were studies have showed that miR-98-5p could inhibit hepatoma cells proliferation while induce cell apoptosis, partly at least, via inhibition of its target gene IGF2BP1(8). Another study suggested that miR-98 plays an anti-invasion role by inhibiting glioma cell migration and invasion and determined that the IκB kinase IKKε is a direct target of miR-98 in glioma cells(9). However, there is not too much research about of miR-98-5p in the process of liver fibrosis. Siragam, V. et al found that the miR-98 inhibited breast cancer cell proliferation, survival, growth, invasion, and angiogenesis by targeting activin receptor-like kinase-4 and matrix metalloproteinase-11(39).Yang, G.et al found that P21-activated protein kinase 1(PAK1) is highly expressed in non-small cell lung cancer, whereas miR-98 is down-regulated(13). They demonstrated that miR-98 directly targets the 3'UTR of PAK1 and is involved in the proliferation, migration, invasion and apoptosis of NSCLC cells. MiR-98 protects endothelial cells against apoptosis induced hypoxia/reoxygenation by targeting caspase-3 in the kidney with ischemia reperfusion injury (IRI)(14). It has been reported that in the process of human intervertebral disc degeneration, miR-98-5p can target the IL-6/STAT3 signaling pathway to promote the degradation of extracellular matrix(40).

Activation of HSC plays an important role in the process of liver fibrosis(35).TGF β signaling plays a central role in the activation of hepatic stellate cells(36). TGFβ1associates with type I (TGFβRI) and type II (TGFβRII) receptor to form a heterotetrametric complex(15; 37).Then, the activated TGFβRI phosphorylates the downstream effectors Smads, which transmit signals from the cell membrane and cytoplasm to the nucleus, and cooperates with other nuclear factors to activate or inhibit the transcription of target genes(38).We found that transfection of miR-98-5p mimic in LX2 cells resulted in a significant down-regulation of TGFβR1 at both protein level and RNA level. In addition, our study confirmed that miR-98-5p can down-regulate the activity of the wild-type TGFβR1 3 'UTR reporter vector by binding to the second site of TGFβR1 3'UTR, whereas when mutant the second binding site of 3' UTR of TGFβR1, the down-regulation activity disappeared. Thus,

miR-98-5p prevents liver fibrosis by targeting TGFβR1, blocking TGFβ1/Smad3 signaling pathway, which play an important role in the progress of liver fibrosis. Those results suggest that miR-98-5p could be a potential therapeutic target for inhibiting liver fibrosis. We also found that the serum levels of miR-98-5p in patients with liver fibrosis were significantly lower than those in healthy controls and HBV carriers. However, there is no difference between healthy controls and HBV carriers. Thus, serum miR-98-5p may represent a potential diagnostic biomarker for liver fibrosis. Our study provides a new way to understand the relationship between miRNAs and hepatic fibrosis and provide a basis for discovering new therapeutic targets.

In conclusions, the expression of miRNAs in patients with liver fibrosis is significantly different from healthy volunteers. Many signal pathways of hepatic fibrosis are regulated by miRNAs. The potential value of miR-98-5p is as diagnostic biomarkers and therapeutic targets for HBV-related liver fibrosis. Future studies will be required to monitor serum miR-98-5p levels in patients to determine the relationship to liver disease development and progression.

Materials and Methods

Participants

In the first stage of microarray analysis, the whole blood was collected from the HBV-related liver fibrosis patients (S2/3, n=8) based on Scheuer's staging criteria in Beijing Ditan Hospital from 2013 to 2014 and from healthy volunteers(n=7) as control group. All subjects must be between the age of 18 and 65, regardless of gender, and able to understand and sign informed consent. The inclusion criteria of liver fibrosis group were required：1) chronic hepatitis B or HBsAg positive for more than 6 months; 2) HBV-DNA > 500IU/ mL without nucleos(t)ide analogues antiviral treatment within 6 months; 3) liver biopsy indicated liver fibrosis staging greater than S1.The exclusion included: 1) fibrosis score of liver biopsy within 6 months was above S3;2) color ultrasound or CT examination suggested signs of cirrhosis; 3) acute and chronic hepatitis with the presence of liver cancer and other non-HBV viruses

infection(including HCV, HDV, CMV)and other liver disease (such as autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, genetic metabolic liver disease, drugs or toxic hepatitis, alcoholic liver disease) or combined with unstable diabetes, high blood pressure, thyroid disease or other serious illnesses such as heart, lung, and kidney disease or infection, etc.

For the analysis of potential correlation between miR-98-5p and liver fibrosis, we enrolled another 70 recruited patients with chronic HBV infection and 29 healthy individuals as controls to conduct real-time QRT-PCR validation of miR-98-5p expression levels.

RNA extraction of plasma

Six milliliter vein blood was collected from the subjects. The samples were centrifuged at 3500 rpm for 15 min. The supernatant plasmas were immediately separated and stored at −80℃. Plasma total RNA was extracted using TRIZOL reagents (Invitrogen, USA). The quality of the extracted RNA was detected by the following methods: 1) Nanodrop detection: The purity of RNA samples (OD260/280≥1.8; OD260 / 230≥1.0).2) Qubit 2.0 Detection: Accurately quantify the concentration of RNA samples (total RNA concentration ≥ 250 ng / ul).3) Agilent 2100 bioanalyzer detection: to detect the integrity of RNA samples, to ensure that the use of qualified samples for sequencing according to previously described (total RNA RIN value ≥ 8.0, 28S/18S ≥ 1.5; map baseline without lifting; 5S peak normal)(41).

IlluminaHiSeq sequencing

After the sample passed the test,1.5μg RNA sample was used as the initial amount, and the volume was replenished with no ribozyme water to 6μl.The small RNA bank was constructed using the small RNA Sample PreKit.T4 RNA Ligase 1 and T4 RNA Ligase 2 were ligated at the 3'end and 5'end of small RNA, respectively. Reverse transcription was used to synthesize cDNA, PCR amplification, and the target fragment was screened by glue separation technique. When bank is qualified, the high-throughput sequencing was performed with HiSeq 2500 as previously described.(42)

Sequencing analysis

Since the differential expression analysis of miRNAs is an independent statistical hypothesis test for a large number of miRNAs expression levels, there will be a problem of false-positive. Therefore, in the analysis process, the Benjamini-Hochberg correction method is sometimes used to estimate the significance P-value, and eventually uses the False Discovery Rate (FDR) as a key indicator of differential expression miRNAs screening. Fold Change (FC) represents the ratio of the expression between two sample groups. In the process of detection, $|\log_2 (FC)| \geqslant 1$; $FDR \leqslant 0.01$ as the screening standard. Based on the results of differential expression and bioinformatics analysis, we selected miR-98-5p for the follow-up mechanisms study. In order to improve the efficiency of prediction, the programs TargetScan Human7.1 and Pic Tar were used to predict the targets of miR-98-5p.

microRNA isolation

Total RNAs were extracted by TRIzol® Reagent (Invitrogen, Thermo Fisher Scientific, USA) and purified by RNeasy MinElute Cleanup kit (QIAGEN, Germany) according to the manufacturer's instructions. To extract miRNAs from serum for QRT-PCR validation, synthetic Caenorhabditis elegans miRNA (cel-miR-39, QIAGEN, Germany) was added and used as the internal control. Purified RNAs were quantified at OD260 and 280 nm using a ND-1000 spectrophotometer (Nanodrop Technology, USA).

Quantitative reverse transcription PCR (QRT-PCR)

MicroRNA expression was measured and quantified using TaqMan MicroRNA Assays kit (Applied Biosystems, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. QRT-PCR reactions were performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA) using a standard protocol. Each sample was run in triplicate. Synthetic cel-miR-39 was used as an internal control gene. The fold expression of the target gene relative to the averaged internal control gene in each sample was calculated using the comparative threshold cycle (Ct) method and evaluated by $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct$ = Patient ($Ct_{\text{miR-98-5p}} - Ct_{\text{cel-miR-39}}$) – Mean of controls ($Ct_{\text{miR-98-5p}} - Ct_{\text{cel-miR-39}}$).

Cell culture

The human HSC cell line LX2 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies, NY, USA), 100 U/mL of penicillin G, and 100μg/mL of streptomycin (Thermo Scientific, IL, USA) at 37℃ in 5% CO_2. miR-98-5p mimic and negative mimic control were purchased from RiboBio Co, Ltd. (Guangzhou, China). Human recombinant transforming growth factor (TGF)-β1 (Peprotech, USA) was used to induce activation of HSCs(43).

RNA extraction, and quantitative real-time-PCR

MiR-98-5p mimic and mimic control were transfected into LX2 cells and RNA was extracted. Total RNA was isolated from cells using Total RNA Kit (Omega, GA, USA). Total RNA was reversely transcribed into single-strand cDNA with PrimeScript® RT reagent Kit (TaKaRa, Beijing, China). qRT-PCR was performed on an ABI 7500 qRT-PCR system (ABI, NY, USA) with SYBR Green PCR Kit (Life Technologies, UK). Relative mRNA amounts were obtained by the $2^{-\Delta\Delta Ct}$method and normalized to endogenous levels of β-actin. The primers used for qRT-PCR are listed in **Table 3** (Sangon, Shanghai, China).

Western blotting

MiR-98-5p mimic and mimic control were transfected into LX2 cells. After 48h, the cells proteins were extracted. Cells were lysed in Mammal Cells Lysis Buffer (Thermo, 78501, USA) containing a protease-inhibitor cocktail (Roche, Germany) and a phosphatase inhibitor cocktail (Roche) for 30 min on ice. Protein concentrations were determined using the Pierce BCA assay (23225, Thermo Scientific). Equal amounts of protein were separated by 12% Bis-Tris Gel/MOPS (NP0341, Invitrogen, USA) and then transferred to a PVDF membrane (ISEQ00010, Millipore, USA) by electroblotting. After blocking with 5% nonfat dry milk (2321000, BD, USA) for about 2h at room temperature. The membranes were incubated overnight at 4℃ with the following primary antibodies: anti-α-smooth muscle actin (α-SMA) (ab5694, Abcam), anti-collagen I (34710,Abcam), anti-collagen III (ab7778, Abcam), anti-Smad3 (9523,CST),anti-P-Smad3 (9520, CST), anti-TGFβR1 (ab31013,Abcam), anti-GAPDH (5174, CST).Membranes were washed 3 times for 10min each with

TBS-Tween. Then the membranes were incubated with secondary anti-bodies goat anti-rabbit (ZB-2301, ZSGB-BIO) or goat anti-mouse (ZB-2305, ZSGB-BIO) for 1h at room temperature. Then the membranes were washed three times as before. Protein bands were detected with an enhanced chemiluminescence system (32209, USA) and the Fusion Solo system (Vilber, France).

Plasmid construction Cloning and mutagenesis of 3'-UTR seed regions

The 3'UTR sequence of TGFβR1 were provided in the NCBI database. As the original 3'UTR sequence was too long (4887bp), and 3'UTR region have two miR-98-5p binding sites. Therefore, the two binding regions were amplified by PCR separately and inserted into thepmirGLO control vector (Promega, E1330). The predicted target site was mutated by site-directed mutagenesis. Genomic DNA was used as a template to amplify the TGFβR1 3'UTR fragment. The primers were introduced restriction endonuclease sites and sequences are listed in **Table 3** (Sangon, Shanghai, China).

Luciferase-reporter assay

LX2 cells were seeded in 48-well plates, transiently transfection was performed with 125ng wild-type (WT) or mutant-type (MUT) reporter plasmids or/and 100mM miR-98-5p mimic or/and mimic control using the jet-PRIMETM transfection reagent (Polyplus-transfection, France), according to the manufacturer's instructions. 25ng of the Renilla luciferase vector (pRL-TK) DNA was also transfected in each well. At 24 hours post-transfection, luciferase activity was measured on a microplate luminometer using Dual-Luciferase Reporter Assay Kit (Promega, USA), according to the manufacturer's instructions.

Statistical analysis

All experiments were performed at least three times. The results are expressed as mean ± standard error of the mean (SEM). Group comparison was performed by paired Student's *t* test. $P<0.05$ was considered statistically significant.

Data Availability

This is a resubmission of an earlier paper. The results/data/figures in this manuscript

have not been published elsewhere, nor are they under consideration by another publisher.

Animal Research (Ethics)

No animal experiments were involved in this study.

Consent to Participate (Ethics)

There is no conflict of interest to disclose. This is noted in the manuscript.

Consent to Publish (Ethics)

All authors agreed for publication in Hepatology International as an Article.

Plant Reproducibility

Clinical Trials Registration

This study has passed the ethical review and obtained the approval of the ethical review（No. 2013-061-2）.

Author Contributions

Y.M. and X.Y. performed the experiments and wrote the manuscript; X.Y., M.H., K.H. and P.L. collected blood samples from the HBV-related liver fibrosis patients; Y.M., X.Y. and M.H. analyzed and interpreted the data; S.L. contributed to scientific discussion; J.C. and H.X. designed the experiments, provided useful advice on the manuscript and modified the manuscript.

Conflict of Interest

The authors declare no competing interests.

Funding

This work was supported by the Beijing Nova Program (No. Z201100006820051 to XY), National Science and Technology Major Project of China(2014ZX10005001 to HX, 2013ZX10002005 to JC), National Science and Technology Major Project of China(2017ZX10202202 to SY, 2018ZX10715-005 to SY), the Beijing municipal science and technology project of traditional Chinese medicine(JJ2014-25), the Seedling raising plan of Beijing Ditan Hospital, Capital Medical University (DTYM201616), Beijing Municipal Administration of Hospitals (No.ZYLX201402,

No. DFL2015170) and Capital's Funds for Health Improvement and Research (CFH 2020-1-2171).

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591 # Figure Legend

592 **Fig.1 Microarray screening for microRNAs associated with liver fibrosis infected**
593 **with HBV**

594 (A)Heatmap showing differentially expressed miRNAs of healthy control group
595 versus the liver fibrosis group (7 versus 8). (B)Target genes GO annotation
596 classification statistical figure of differentially expressed the miRNA. (C) Target gene
597 COG annotation of differentially expressed of miRNA. (D)Target gene KEGG
598 classification map of differentially expressed miRNA.

599 **Fig.2 The expression level of miR-98-5p is reduced in the liver fibrosis group**

(A)Heatmap showing 26 down-regulated miRNA in liver fibrosis group versus healthy control group and the target genes GO annotation classification. (B) The expression of miR-98-5p in the peripheral blood of healthy group and liver fibrosis group. Data are mean ± SEM of three independent experiments (***p < 0.001).

Fig.3 Effect of miR-98-5p on migration and proliferation of LX2 cells

(A) Over-expression miR-98-5p mimic in LX2 cells resulted in significantly suppressing the wound healing and migration. (B) miR-98-5p inhibitor led to acceleration of the wound healing and migration in LX2 cells. (C) Over-expression miR-98-5p mimic in LX2 cells resulted in significantly suppressing LX2 cells proliferation in 24 h, 48 h and 72h. (D) miR-98-5p inhibitor led to increasing proliferation of LX2 cells (*P<0.05, **P<0.01, ***P<0.001).

Fig.4 Effect of miR-98-5p on TGFβ1/Smad3 signaling pathway

(A) LX2 cells were transfected with miR-98-5p mimics cultured for 72 h and detected the mRNA levels of α-SMA, collagen1A1, collagen1A2, collagen3A1, collagen5A1, Smad3, p-Smad3 and TGFβRI by qRT–PCR. The mRNA levels were normalized against β-actin and the results are shown as foldchange compared with LX2 cells mimic control transfection. Data are mean ± SEM of three independent experiments (***p < 0.001). (B) LX2 cells were transfected with miR-98-5p mimics cultured for 48 h and detected the protein levels of α-SMA, collagen I, collagen II, p-Smad3 Smad3 and TGFβRI by western blot.

Fig.5 MiR-98-5p directly targets TGFβRI

(A) Predicted binding sequences between miR-98-5p and seed matches TGFβRI.

(B, C) Luciferase reporter vectors were generated by inserting the wild-type (WT) or mutated (Mut)3'UTR fragments of TGFβRI into pmirGLO plasmid. Luciferase reporter assays at 24 h after transfection with wild-type or mutated plasmids, co-transfected with mimic control or miR-98-5p mimic. Data shown are means ± SEM of independent experiments (n=3, ***P < 0.01).

Fig.6 The expression level of miR-98-5p

The expression level of miR-98-5p in the peripheral blood of healthy group, HBV carrier group and liver fibrosis group. Data are mean ± SEM of three independent

experiments (*p < 0.05).

Tables

Table 1. Clinical characteristics of patients with liver fibrosis and healthy controls (HCs)

Index	Liver fibrosis(S2/3)	Healthy control
sex（M/F）	5/3	4/3
age（years）	38.75±3.80	35.57±3.18
ALT（U/L）	69.23±17.55	11.22±1.46
AST（U/L）	36.65（24.68，102.07）	15.00±0.84
TBiL（μmol/L）	13.4（10.82, 16.97）	12.1 (6.9，15.6)
DBiL（μmol/L）	4.825±0.65	4.8 (3.8，6.2)
TP（g/L）	73.87±2.35	74 (71.3,78.5）
ALP（g/L）	56.25（52.6，79.75）	——
GGT（U/L）	28.45（15.25，50.05）	——
HBV-DNA（IU/mL）	2.47E+5（1.79E+4，1.07E+6）	——
HBeAg +（%）	50	——

The normal distribution data are expressed as $x \pm s$, and the nonconforming data are expressed by the median (P25, P75).

Table 2. Clinical characteristics of patients with chronic HBV infection and healthy controls (HCs)

Index	HBV infection		Healthy controls (n=29)	P-value HBV vs Healthy controls
	Liver cirrhosis (n=43)	HBV carrier (n=27)		
Gender（M/F）	32/11	12/16	9/20	0.98
Age（years）	52 (17)	44 (15)	54 (15)	0.98
ALT（U/L）	28.9（20.1）	19.6 (10.9)	<40.00	
AST（U/L）	26.8（7.4）	19.5 (5.35)	<35.00	
TBiL（μmol/L）	12.0 (9.4)	12.0 (5.25)	<18.80	
ALB（g/L）	46.8 (3.9)	48.5 (3.35)	<40.00	
ALP（g/L）	84.3（29.8）	62.25 (25.4)	N/A	
GGT（U/L）	26.4（21.2）	13.3 (7.45)	N/A	
HBV-DNA (IU/ml)	2.99E+4（2.82E+4）	2.67 E+4(1.03E+4)	N/A	
HBeAg +（%）	69.77	N/A	N/A	
HBsAg (IU/ml)	3.52 (0.76)	2.98 (1.21)	N/A	

For age, ALT, AST, TBiL, ALB, ALP, GGT, HBsAg, HBV DNA titers and HBsAg, data are

presented as median (interquartile range). P-values <0.05 are considered as significant. N/A, not available. Calculated by Fisher's exact test for gender distribution. Calculated by Mann-Whitney U-test for age.

Table 3. Primers Used for Real-Time Polymerase Chain Reaction (PCR)

Genes	Sense（5'-3'）	Antisense（5'-3'）
α-SMA	GGGAATGGGACAAAAAGACA	CTTCAGGGGCAACACGAA
Smad3	CACCACGCAGAACGTCAA	GATGGGACACCTGCAACC
Collagen 1A1	GGGATTCCCTGGACCTAAAG	GGAACACCTCGCTCTCCA
Collagen 1A2	CTGGAGAGGCTGGTACTGCT	AGCACCAAGAAGACCCTGAG
Collagen 3A1	CTGGACCCCAGGGTCTTC	GACCATCTGATCCAGGGTTTC
Collagen 5A1	CCTGGATGAGGAGGTGTTTG	CGGTGGTCCGAGACAAAG
TGFβR1	GCTTAGGGGTGTGGGTCTTC	AAGCCAAGTTTTCACCCCCA
wt-UTR-1	gctagcTTCTACAGCTTTGCCTGAACTCTCC	ctcgagCATTGTAATTCAGCAATCCAACTC
wt-UTR-2	gctagcATCCCATTATGCAATCTTGTTTGTA	ctcgagGGCAGAGATTACACTGATAAAGCC
mut-UTR-1	AGGTCAATTGTTGTTCGTGACTGAGAGGGA	TCCCTCTCAGTCACGAACAACAATTGACCT
mut-UTR-2	GGTACATTAAGCACTTTGTGTATAT	ATATACACAAAGTGCTTAATGTACC

Figures

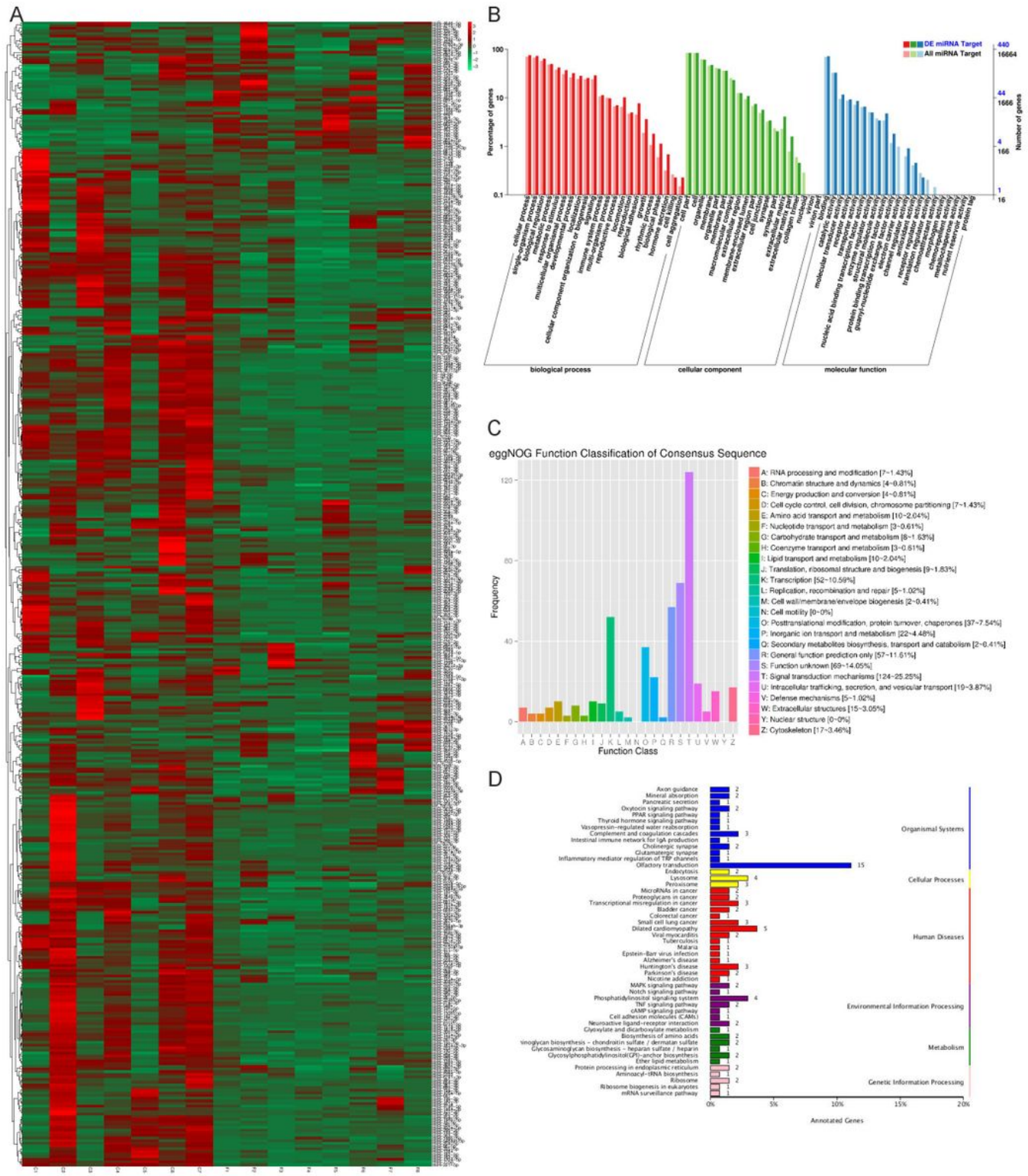

Figure 1

Microarray screening for microRNAs associated with liver fibrosis infected with HBV (A)Heatmap showing differentially expressed miRNAs of healthy control group versus the liver fibrosis group (7 versus 8). (B)Target genes GO annotation classification statistical figure of differentially expressed the miRNA.

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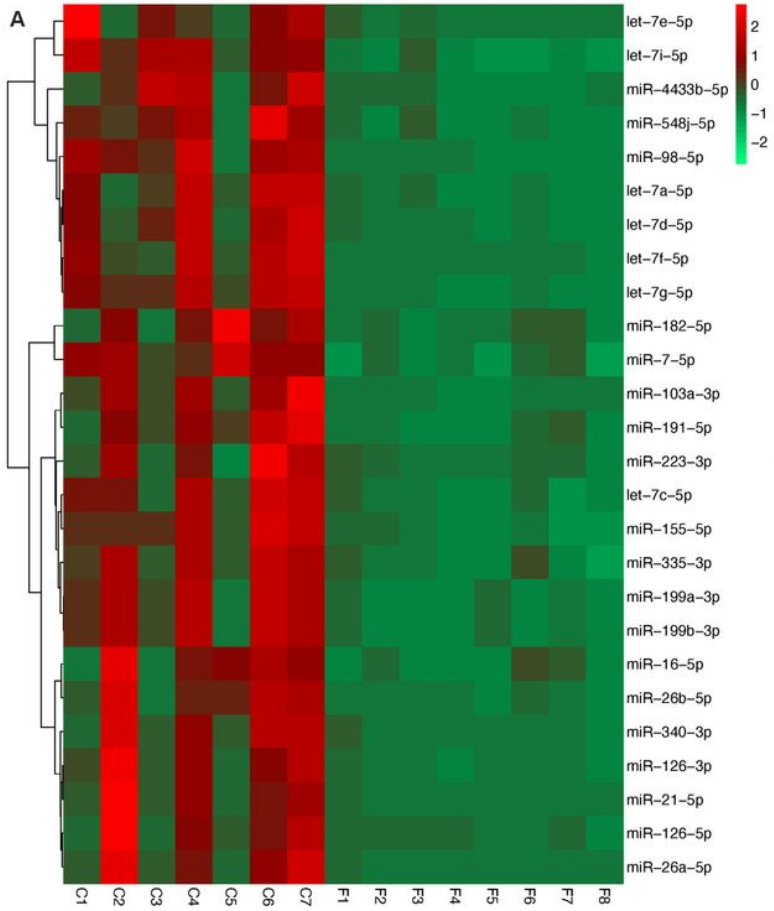

GO Term	P-value
collagen type IV	3.892119e-05
basal plasma membrane	1.669471e-04
apoptotic cell clearance	1.846329e-04
IgG receptor activity	4.344866e-04
antibody-dependent cellular cytotoxicity	4.560354e-04

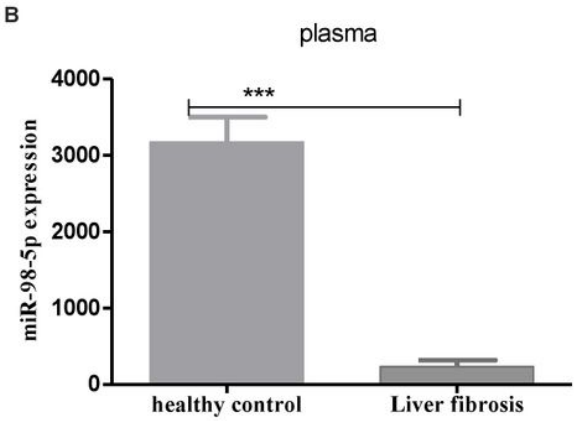

Figure 2

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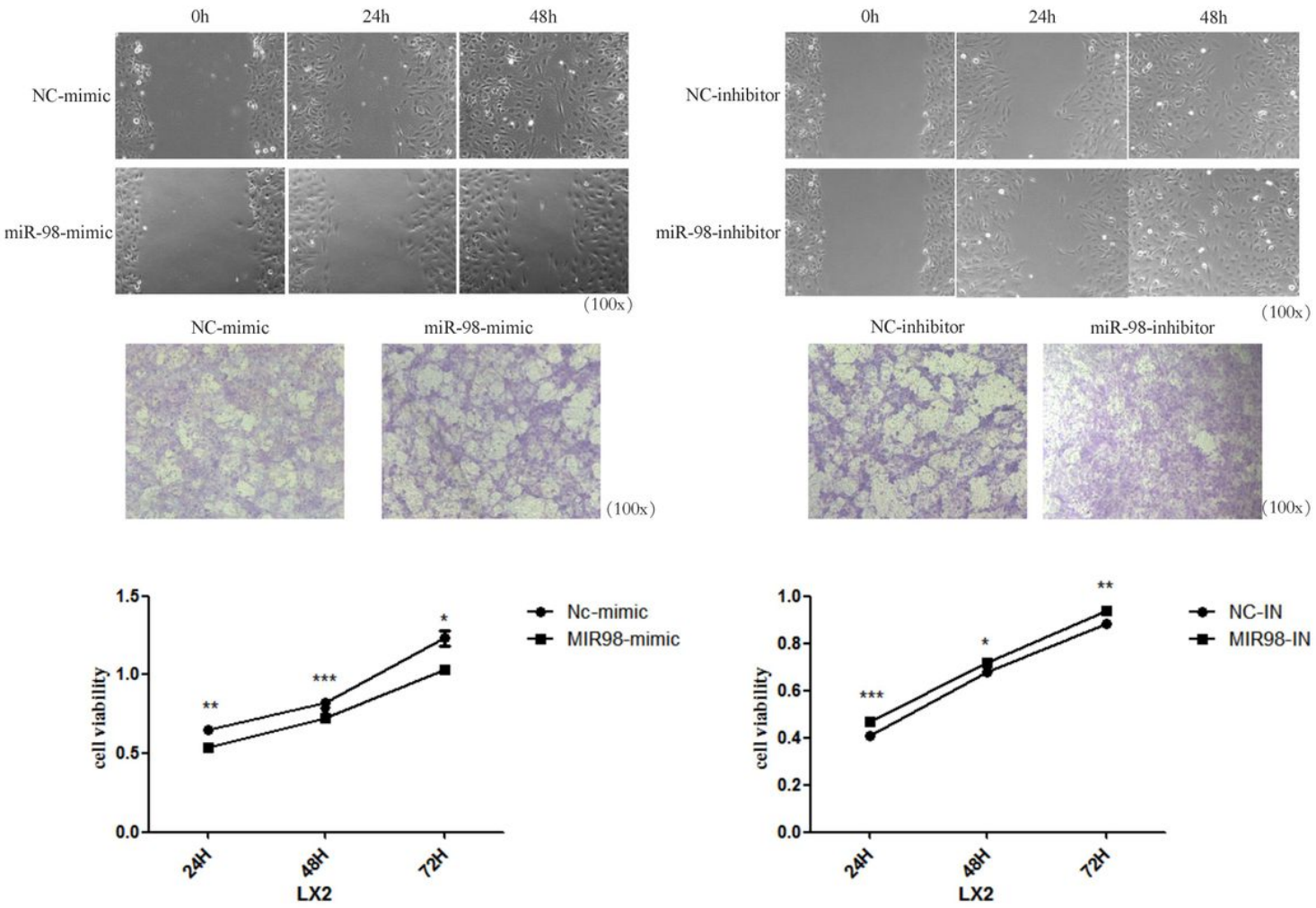

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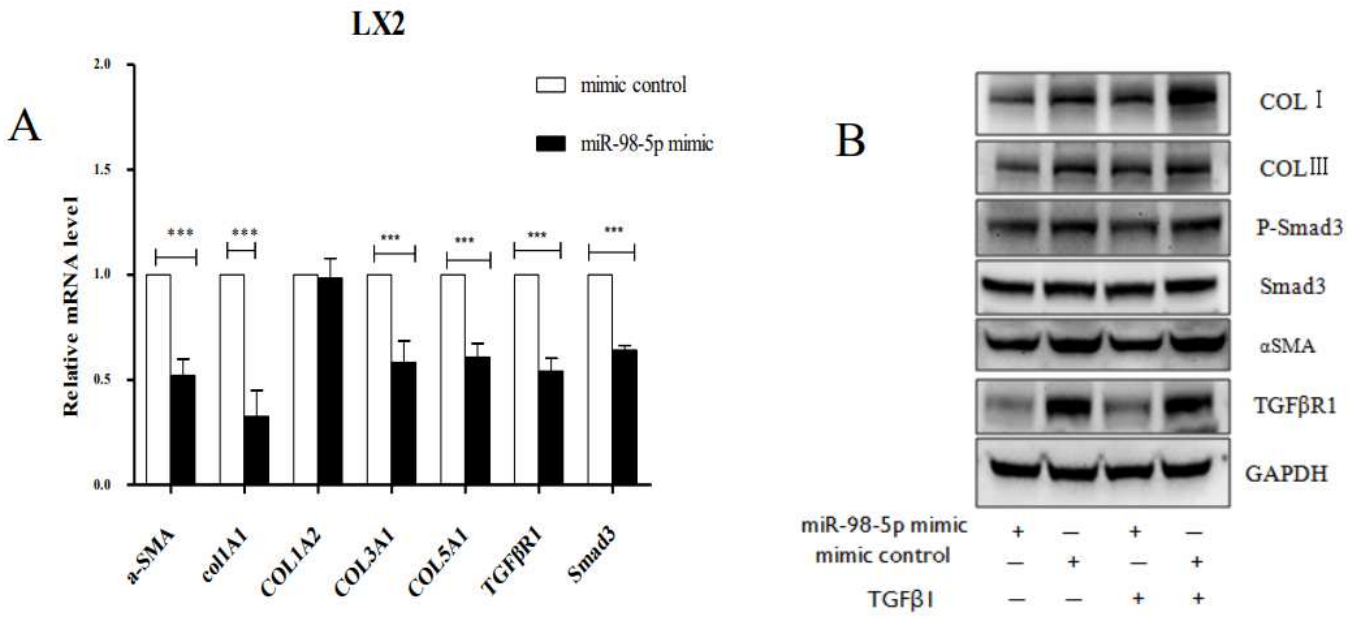

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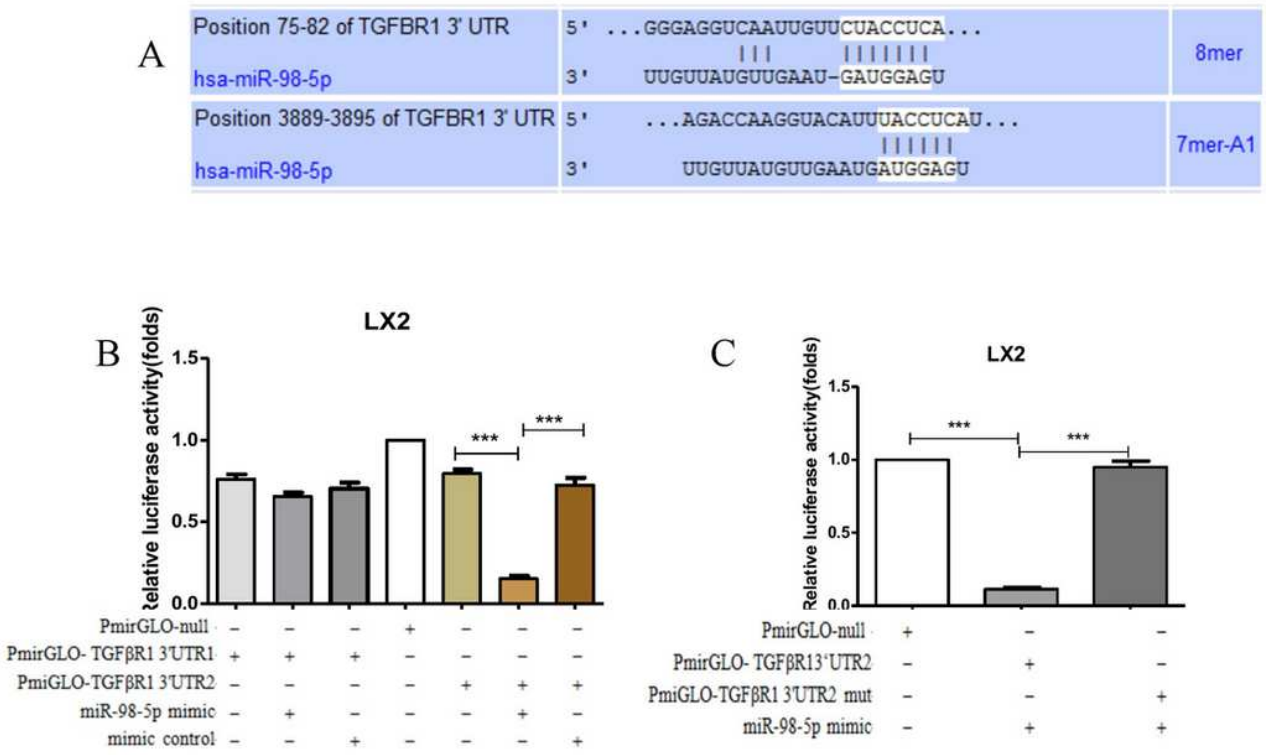

Figure 5

MiR-98-5p directly targets TGFβRI (A) Predicted binding sequences between miR-98-5p and seed matches TGFβRI. (B, C) Luciferase reporter vectors were generated by inserting the wild-type (WT) or mutated (Mut)3'UTR fragments of TGFβRI into pmirGLO plasmid. Luciferase reporter assays at 24 h after transfection with wild-type or mutated plasmids, co-transfected with mimic control or miR-98-5p mimic. Data shown are means ± SEM of independent experiments (n=3, ***P < 0.01).

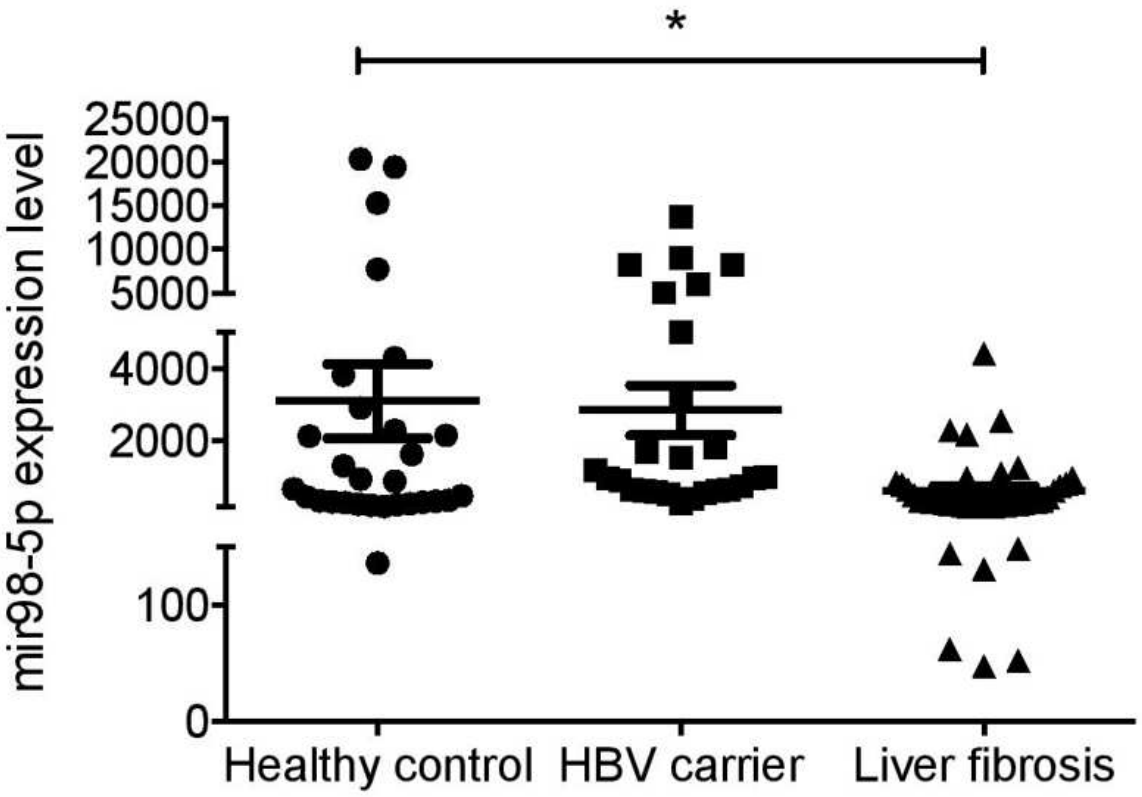

Figure 6

The expression level of miR-98-5p The expression level of miR-98-5p in the peripheral blood of healthy group, HBV carrier group and liver fibrosis group. Data are mean ± SEM of three independent experiments (*$p < 0.05$).