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

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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 (miRNA) 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 (F2/3, n=10) based on Scheuer's staging criteria. In addition, healthy volunteers (n=8) 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 carried out. Gene ontology (GO) enrichment analysis and KEGG pathway enrichment analysis of differentially expressed miRNAs target genes were performed. Compared with the healthy control group 104 miRNAs were screened out from the liver fibrosis group, among which 72 miRNAs were up-regulated and 32 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 TGFβR1 and blocking TGFβ1/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 microRNAs 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). The aim of this study was to detect plasma miRNA biomarkers associated with fibrosis in patients with chronic HBV infection 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. MiR-98-5p was selected according to bioinformatics and was discussed its mechanism in the progress of hepatic fibrosis. A study says that miR-98-5p could inhibit hepatoma cells proliferation while induce cell apoptosis, partly at least, via inhibition of its target gene IGF2BP1(7). 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(8). Currently, there are not many studies on the role of miRNA in liver fibrosis. 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.

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. Here, 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.

Results

Patient information

There was no significant difference in age and sex between the liver fibrosis group and the healthy group. The detailed clinical data of the two groups were presented in Table 1 and Table 2.

Bioinformatics Analysis

Compared with the healthy control group, 104 miRNAs were screened out from the liver fibrosis group, among which 72 miRNAs were up-regulated and 32 were down-regulated. 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. 1)**. Pathway annotations for the target genes of the miRNAs identified were found that it participated in many signal including MAPK signaling pathway, TNF signaling pathway, Notch signaling pathway, phosphatidylinositol signal system and so on **(Fig. 2, 3)**.

The expression level of miR-98-5p

Compared with the healthy group, the expression of miR-98-5p in the peripheral blood of liver fibrosis group was significantly lower ($P < 0.001$) **(Fig. 4).**

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

miR-98-5p suppress the expression of TGFβR1

To explore whether miR-98-5p are involved in regulating TGFβRI expression, we integrated the results of the prediction software programs Target Scan and PicTar. MiR-98-5p predicted to target TGFβRI, were found by all two programs. We all know that miR-98-5p was down-regulated in liver fibrosis. 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. 6A)**. The proteins of α-SMA, collagen Ⅰ, collagen Ⅲ, p-Smad3 were significantly reduced when LX2 cells were transfected with miR-98-5p.The level of Smad3 was not obvious. To further explore the mechanism underlying miR-98-5p regulation of HSC 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. 6B)**. 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

MiR-98-5p suppress the expression of TGFβR1 in both mRNA and protein level. And

miR-98-5p has potential binding sequences in the 3'UTRs of TGFβR1 **(Fig.7A)**. 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. 7B, 7C)**. These results strongly indicate that TGFβR1 are direct targets of miR-98-5p.

The mechanisms of miR-98-5p as a potent liver fibrosis suppressor

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.

Mir-98-5p is 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. 8, $P<0.05$)**. Interestingly, there is 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(9). 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(10). Many studies tried developing non-invasive tests to substitute liver biopsy for fibrosis assessment(11). 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(12). 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(13). Guo et al found that 12 miRNAs were upregulated, and 9 miRNAs were downregulated in activated HSCs compared with rat quiescent HSCs(14). Our study found that compared with the healthy control group, 104 miRNAs were screened out from the liver fibrosis group, among which 72 miRNAs were up-regulated and 32 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.

Activation of HSC plays an important role in the process of liver fibrosis(15). TGF β signaling plays a central role in the activation of hepatic stellate cells(16). TGFβ1 associates with type I (TGFβRI) and type II (TGFβRII) receptor to form a heterotetrametric complex(17; 18). 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(19). Among the differentially expressed miRNAs, the expression of miR-98-5p was significantly reduced in the liver fibrosis group. Bioinformatics predicts that miR-98-5p can target TGFβR1 and block TGFβ1 / Smad3 signaling pathway.

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(20).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 (21). 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)(22). 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 (23).

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, confirming that miR-98-5p down-regulates the mechanism of hepatic fibrosis-related genes by down-regulating TGFβR1. MiR-98-5p by blocking the expression of TGFβR1, blocking TGFβ1 / Smad3 signaling pathway, and then play the role of anti-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 ofmiR-98-5pis 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 (F2/3, n=10) in Beijing Ditan Hospital from 2013 to 2014 and from healthy volunteers(n=8) as control group. The inclusion criteria of liver fibrosis group were chronic hepatitis B or HBsAg positive for more than 6 months and diagnosis by liver biopsy without therapy. The exclusion included: history of treatment for hepatic disease, liver cancer, chronic HCV or HIV infection, and Patients with chronic liver disease attributed to causes other than HBV infection.

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 was 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 (total RNA RIN value ≥ 8.0, 28S/18S ≥ 1.5; map baseline without lifting; 5S peak normal).

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.

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.

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 the pmirGLO 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 as follows:

The first site(UTR1-362bp)：

Wt-Up (NheI-UTR-1)：5'-gctagcTTCTACAGCTTTGCCTGAACTCTCC-3'

Wt-Down (XhoI-UTR-1)：5'-ctcgag CATTGTAATTCAGCAATCCAACTC-3'

The second site (UTR2-478bp)：

Wt-Up (NheI-UTR-2): 5'-gctagc ATCCCATTATGCAATCTTGTTTGTA-3'

Wt-Down (XhoI-UTR-2): 5'-ctcgag GGCAGAGATTACACTGATAAAGCC-3'

Site mutation:

mut- UTR1-Down 5'-TCCCTCTCAGTCACGAACAACAATTGACCT-3'

mut- UTR1-up 5'-AGGTCAATTGTTGTTCGTGACTGAGAGGGA-3'

mut- UTR2-Down 5'-ATATACACAAAGTGCTTAATGTACC-3'

mut- UTR2-up 5’-GGTACATTAAGCACTTTGTGTATAT-3’

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 isn’t 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.

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Figure Captions

Fig.1 Target genes GO annotation classification statistical figure of differentially expressed the miRNA

The abscissa indicates the GO classification, the right of vertical axis is number of genes, and the left is the percentage of gene number. The significant differences between all miRNA target and differentially expressed (DE) miRNA target, and it is important to analyze whether this function is related to the difference. The target genes of differentially expressed miRNAs were mainly involved in biological processes, such as biological adhesion, cell aggregation and locomotion, etc. Involved in cell components, such as the nucleoid, collagen trimer and extracellular matrix, etc. Involving molecular functions, such as translation regulator activity, channel regulator activity and electron carrier activity, etc.

Fig.2 Target gene COG annotation of differentially expressed of miRNA

The abscissa indicates the contents of the COG classification, and the ordinate indicates the number of genes. The target genes of differentially expressed mainly involved in transcription, general function prediction and signal transduction mechanisms, etc.

Fig.3 Target gene KEGG classification map of differentially expressed miRNA

The ordinate is the name of the KEGG metabolic pathway, and the abscissa is the number of genes and the proportion of the total number of genes on which they are annotated. Target genes participated in many signal pathways including MAPK signaling pathway, TNF signaling pathway, Notch signaling pathway, phosphatidylinositol signal system and so on.

Fig.4 The expression level of miR-98-5p

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.5 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.6 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 fold change 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 III, p-Smad3 Smad3 and TGFβRI by western blot.

Fig. 7 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.8 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
sex（M/F）	7/3	5/3
age（years）	37.38±3.30	45.42±3.46
ALT（U/L）	63.14±46.54	11.26±3.59
AST（U/L）	36.25（27.83，68.83）	15.06±2.08
TBiL（μmol/L）	13.4（12.4,18.4）	11.95(7.68，16.45)
DBiL（μmol/L）	5.26±1.67	4.65(3.93，7.40)
TP（g/L）	76.04±7.59	74.2（71.75,78.45）
ALP（g/L）	68.05（54.2，102.05）	——
GGT（U/L）	34.35（19.95，61.58）	——
HBV-DNA（IU/mL）	2.47E+5（3.94E+4，6.78E+5）	——
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 (log_{10} copies/ml)	2.99（2.82）	2.67 (1.03)	N/A	
HBeAg +（%）	69.77	N/A	N/A	
HBsAg (log_{10} 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

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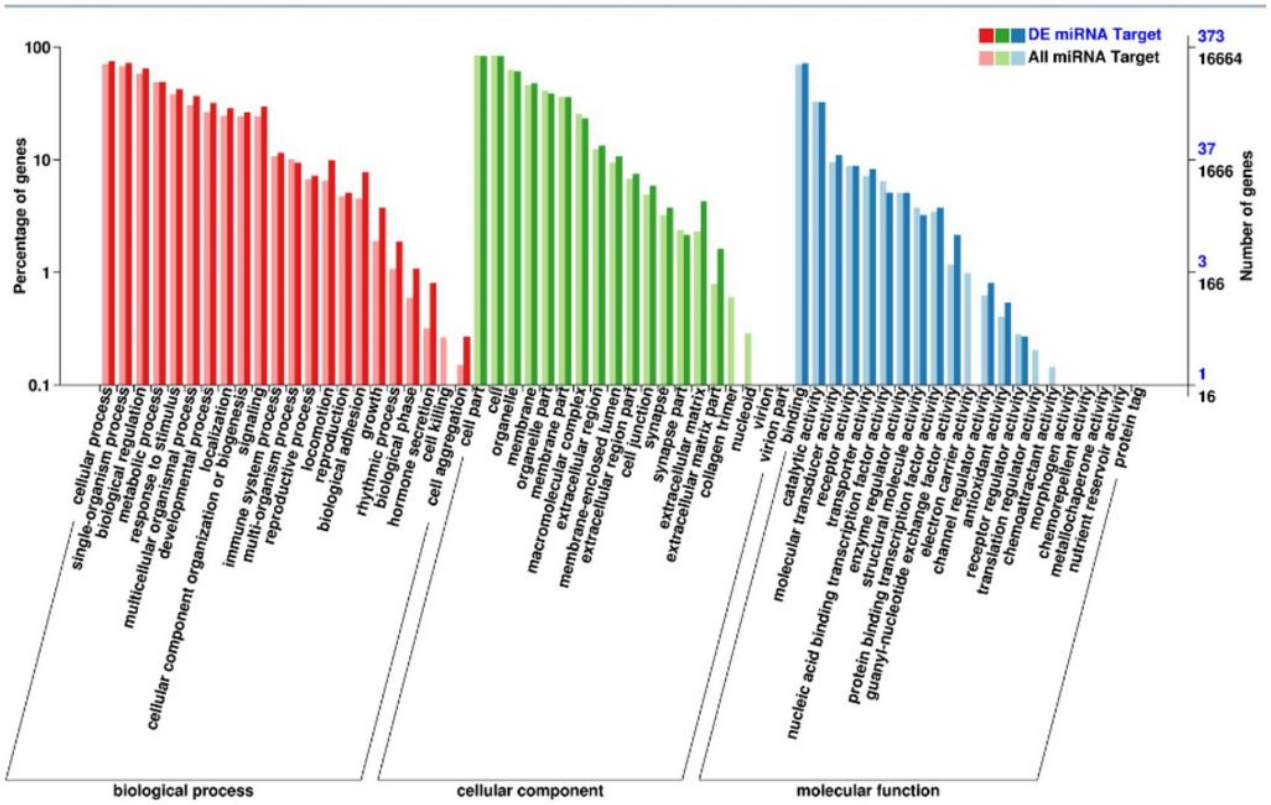
Figures



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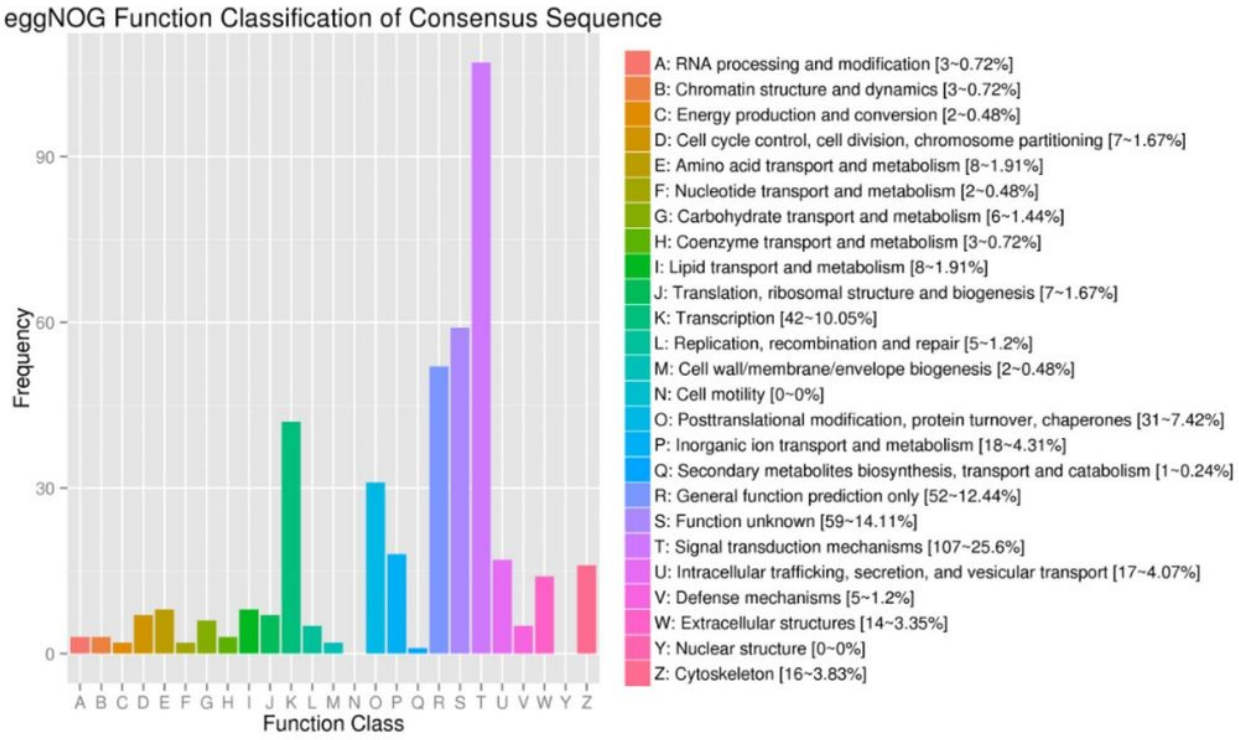

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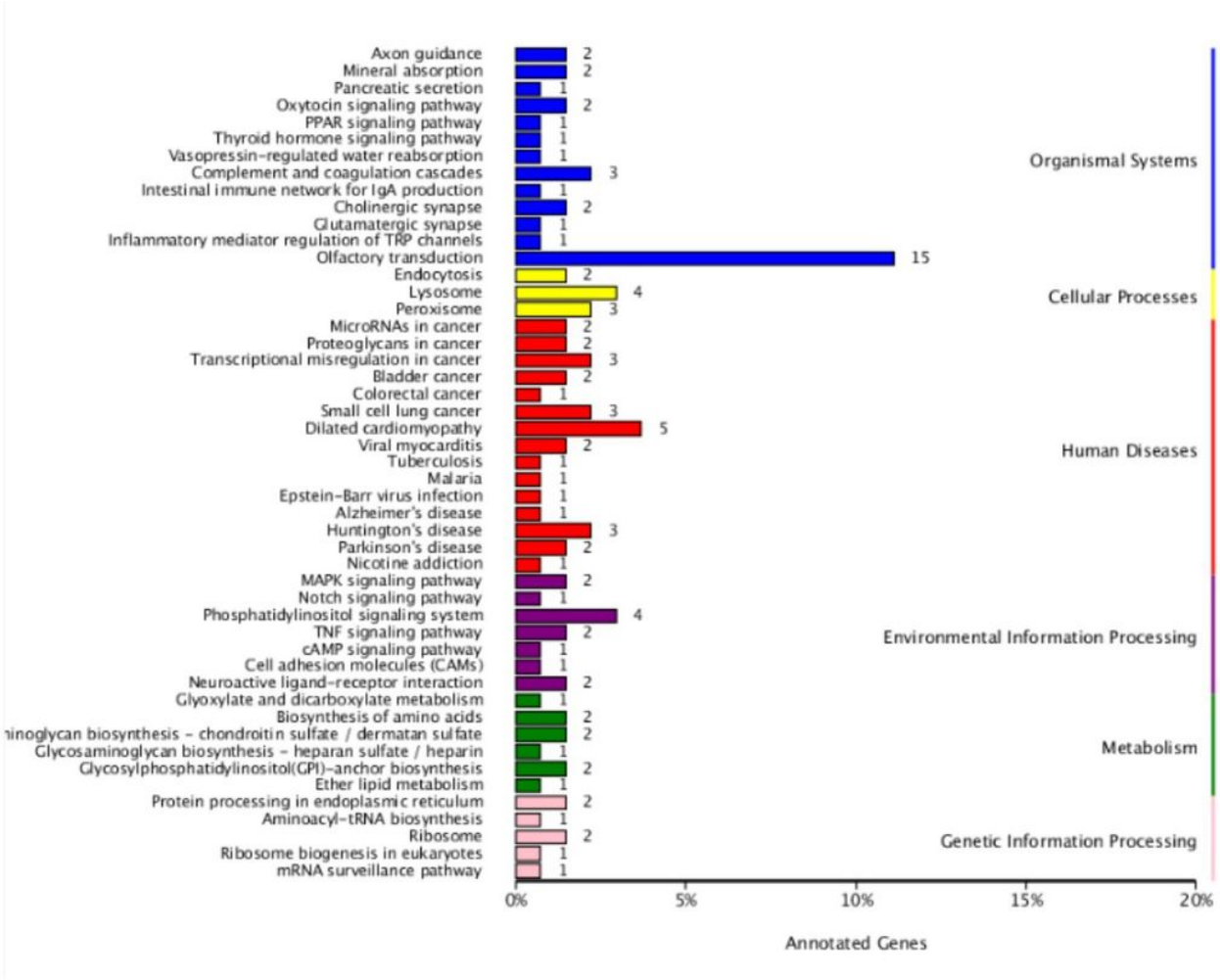

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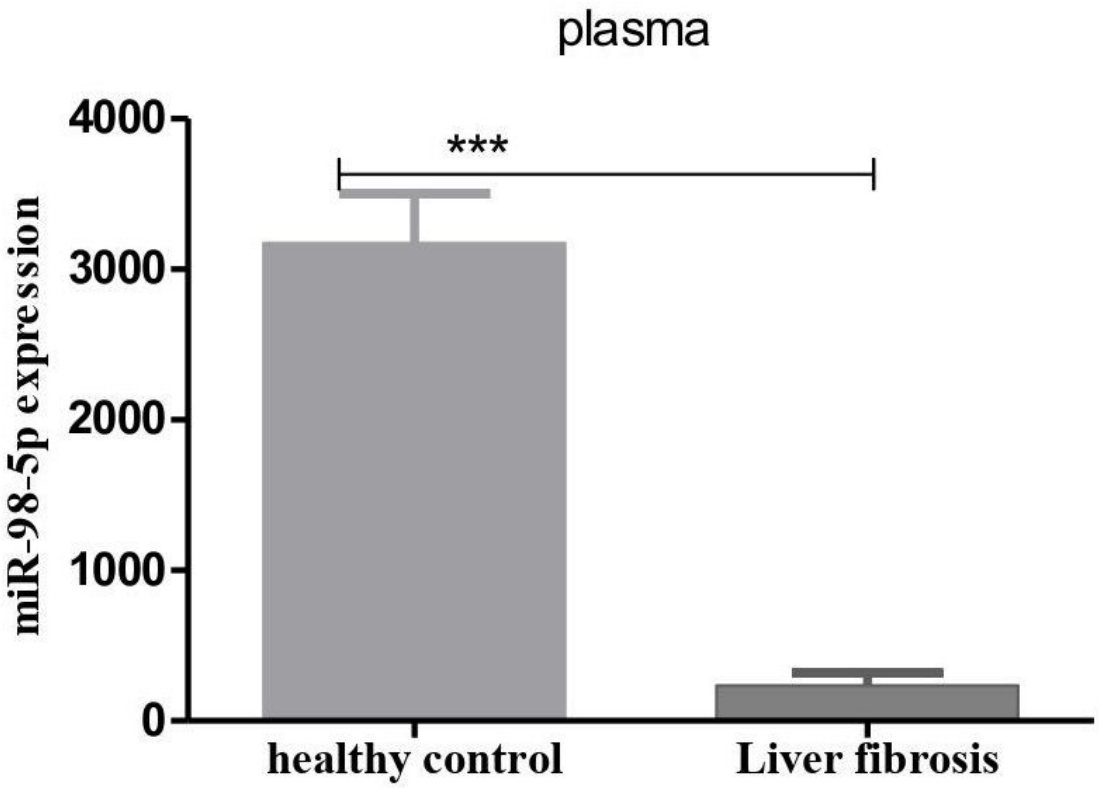

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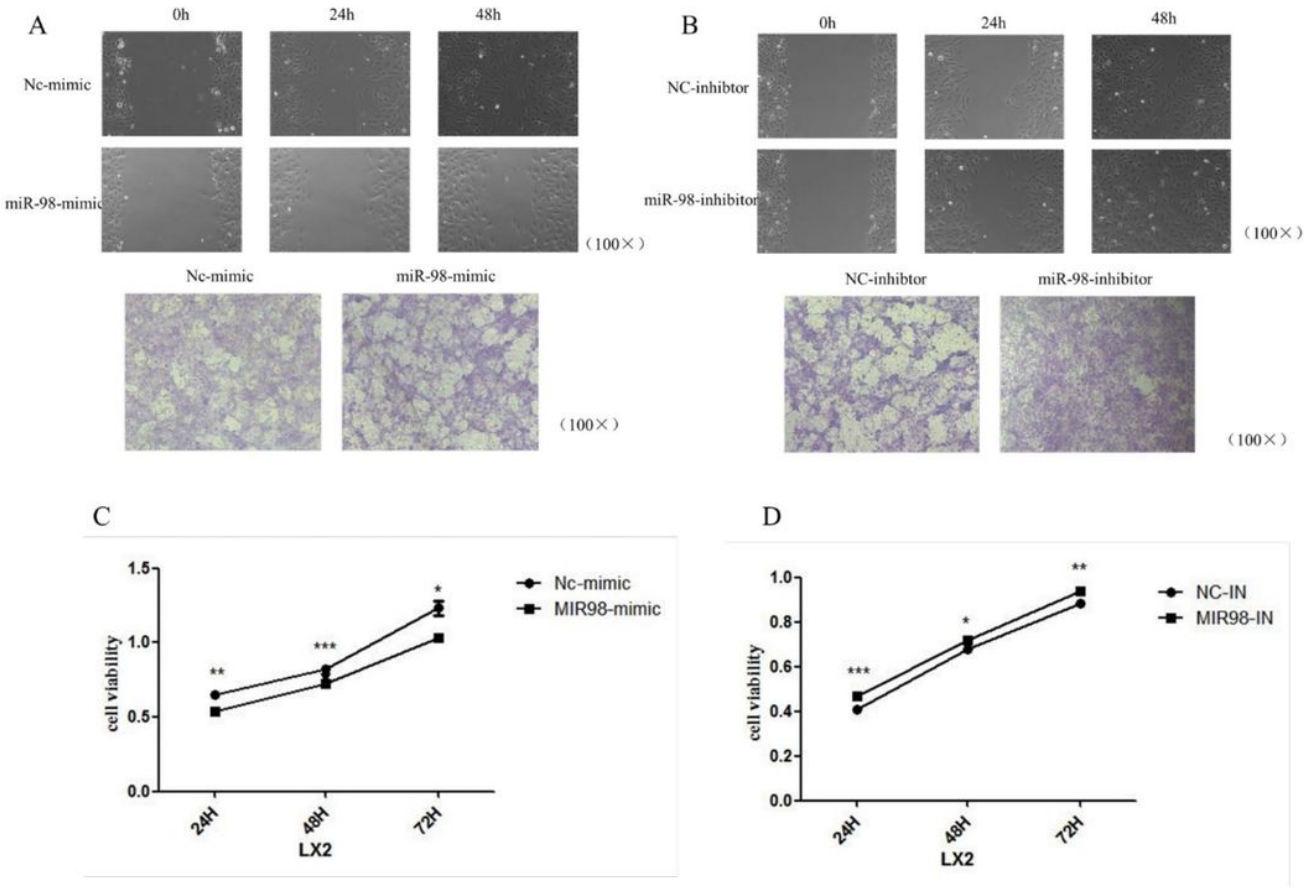
The expression level of miR-98-5p The expression of miR-98-5p in the peripheral blood of healthy group and liver fibrosis group. Data are mean ± SEM of three independent experiments (***p < 0.001).



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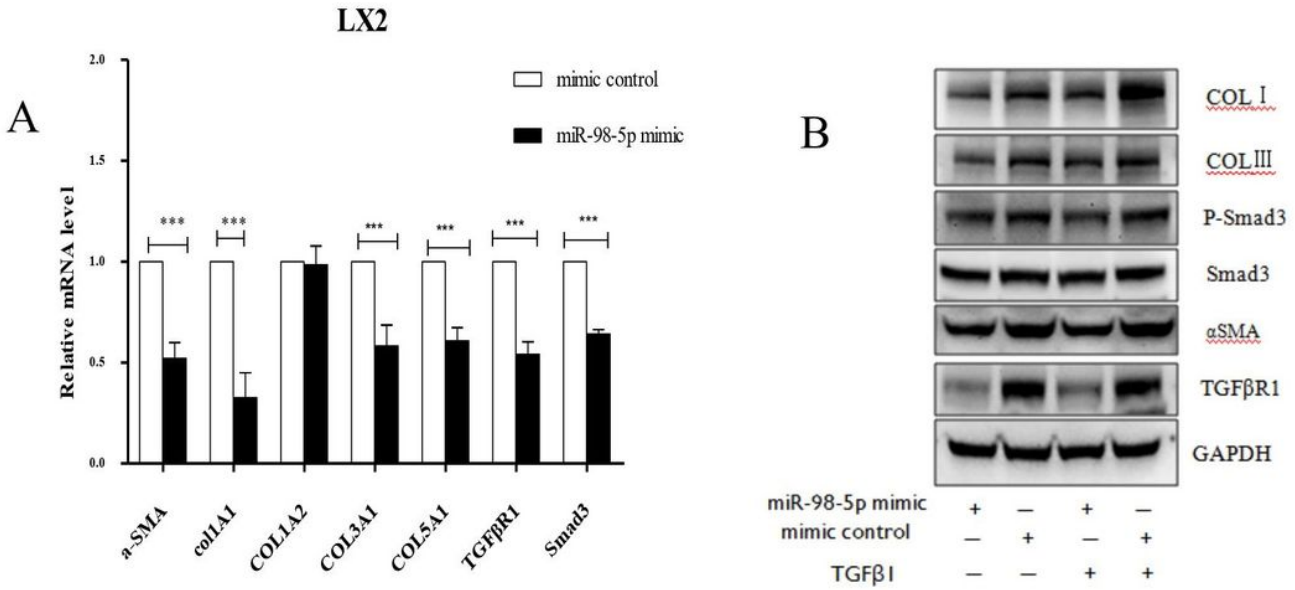

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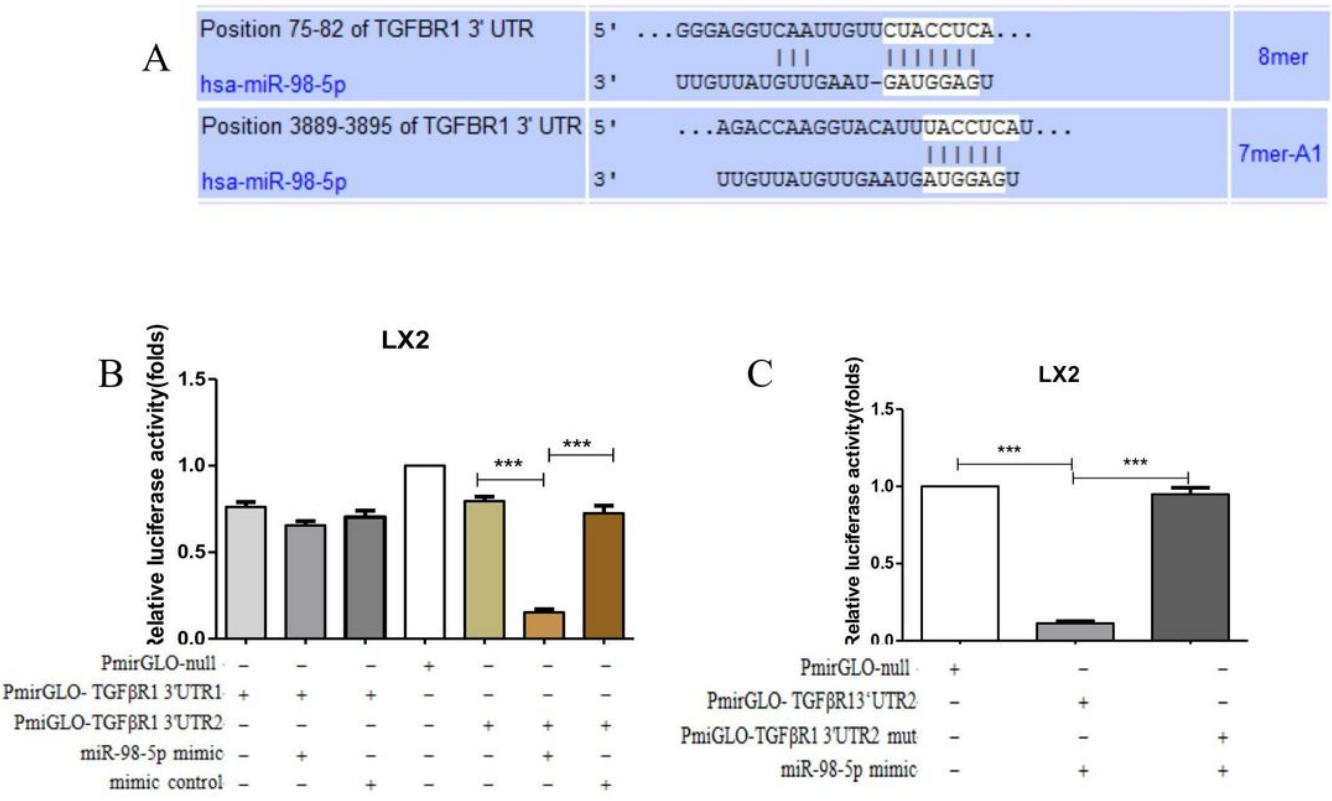

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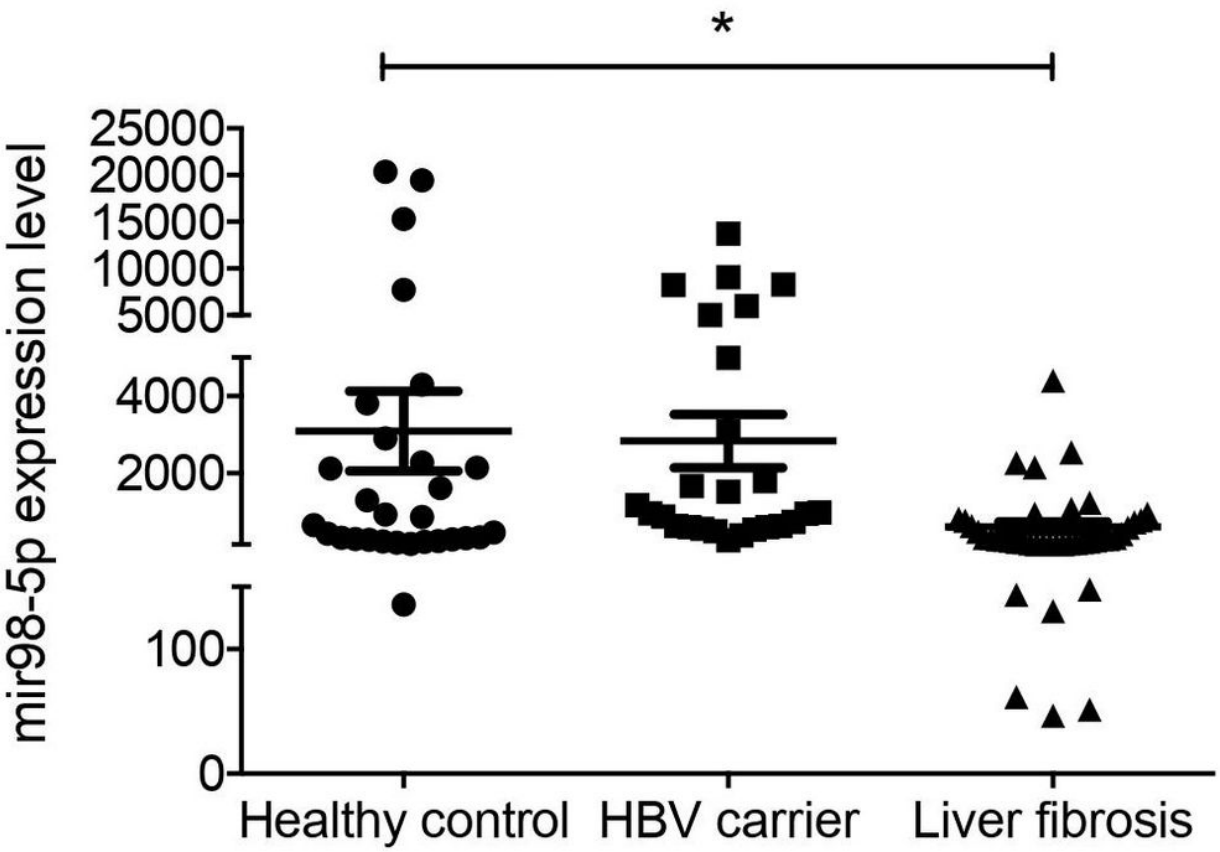

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