MiR-10a improves hepatic fibrosis by regulating the TGFβl/Smads signal transduction pathway

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Abstract. The aim of the present study was to examine the expression variation of the mouse hepatic fibrosis tissue transforming growth factor (TGF)-βl/Smads signal transduction pathway and its correlation with progression of hepatic fibrosis. The promotion effect of microRNA (miR)-10a on hepatic fibrosis and its possible mechanism was also assessed. Forty healthy female 8-week-old C57BL6/J mice were randomly divided into the control group (intraperitoneal injection of 5 μl/g normal saline, twice per week for 8 weeks) and the hepatic fibrosis group (intraperitoneal injection of 5 μl/g 10% CCl_4 olive oil, twice per week for 8 weeks), with 20 mice per group. RT-PCR was used to test miR-10a expression in cells in the control and hepatic fibrosis groups. Cell culture and transfection of miR-10a mimics were conducted in the two groups and a Cell Counting Kit-8 was used to test the expression of TGF-β1 and Smad7 in hepatic fibroblasts. It was found that in comparison with the control group, miR-10a expression was significantly increased in the hepatic fibrosis group compared with the control group (P<0.05). The expression quantity of miR-10a was significantly increased in the transfection group compared with the control group (P<0.05). A high expression of miR-10a significantly improved TGF-β1 expression and reduced Smad7 expression in the hepatic fibrosis group (P<0.05). In conclusion, miR-10a expression was high in mouse hepatic tissues, transfection of miR-10a mimics significantly promoted the cell proliferation of hepatic fibrosis, and miR-10a improved hepatic fibrosis by regulating the TGF-βl/Smads signal transduction pathway.

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Introduction

Hepatic fibrosis is a type of repairing reaction to chronic liver injuries caused by various pathogens, and is a reversible pathological process (1). The removal of relevant pathogenic factors or effective prevention can improve the degree of hepatic fibrosis, otherwise hepatic fibrosis can develop into decompensated liver cirrhosis at the terminal stage. MicroRNA (miR) is a type of endogenous non-coding single-stranded micromolecule RNA with a size of 19-22 nucleotides, and through its complete or incomplete complementary pairing with target mRNA, causes mRNA degradation or translation inhibition, regulating the gene expression level after transcription (2). Previous findings showed that some miRs have a relatively rich expression in liver, and levels of miRs were markedly altered in liver diseases, thus affecting the genesis and development of hepatic diseases (3). miR-10a is involved in multiple physiological and pathological processes including hematopoietic cell differentiation, tumorigenesis and development, and immunoregulation (4,5).

In pulmonary fibrotic mouse lung tissues caused by bleomycin, 161 miRs of different expressions were found, with miR-10a participating in fibroblast activation and collagen deposition by regulating the TGF-β signaling pathway (6). Activation of hepatic stellate cell (HSC) or its phenotypic transformation into myofibroblast is a key link for the formation of hepatic fibrosis (7). Transforming growth factor (TGF)-β1 is a strong cell factor that induces hepatic fibrosis (8), and Smad protein is a key active substrate of TGF-β1 (9). Smad and TGF-β1 cause HSC activation, and initiate collagen gene expression, resulting in the genesis of hepatic fibrosis. Activation of the TGF-β1/Smads signal transduction pathway leads to pathological changes, which play a significant role in the genesis and development of hepatic fibrosis (10).

In the present study, the molecular mechanism of miR-10a in regulating hepatic fibrosis was examined by using miR-10a as a drug target of hepatic fibrosis to guide the clinical diagnosis and treatment of hepatic fibrosis.

Materials and methods

Establishment of hepatic fibrosis mouse model. Forty healthy 8-week-old C57BL6/J female mice, weighing 18-22 g, were

selected in the present study. The animals were randomly divided into the control group (intraperitoneal injection of 5 μl/g normal saline, twice per week for 8 weeks) and hepatic fibrosis model group (intraperitoneal injection of 10% CCI_4 olive oil, twice per week for 8 weeks), with 20 animals in each group fed for 8 weeks and subsequently sacrificed.

The study was approved by the ethics committee of Wenzhou Medical University (Zhenjiang, China).

Preparation of samples. The experimental animals were fasted for 12 h, and sacrificed under ether anesthesia. Blood samples were obtained and 10% neutral formalin was fixed in hepatic tissues to prepare the pathological sections. The remaining hepatic tissues were rapidly frozen in liquid nitrogen, and then stored at -80˚C.

Main reagents. Pronase E was purchased from Merck Millipore (Darmstadt, Germany), collagenase II from Sigma-Aldrich (St. Louis, MO, USA), lymphocyte separation medium from the Institute of Biomedical Engineering of the Chinese Academy of Medical Sciences (Beijing, China), Dulbecco's modified Eagle's medium (DMEM) culture solution dry powder from Gibco (Grand Island, NY, USA), fetal calf serum (FCS) from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Zhejiang, China), INTERFERin siRNA transfection reagent from Polyplus-Transfection (New York, NY, USA), the miRNA analysis system from Applied Biosystems Life Technologies (Foster City, CA, USA), PrimeScript RT-PCR reverse transcription kit from Toyobo (Osaka, Japan), miR-10 mimics from Guangzhou Ruibo Biotechnology Co., Ltd. (Guangzhou, China), all McAbs from Abcam (Cambridge, MA, USA), and the cell lysis solution from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Mouse HSC separation method. After an intraperitoneal injection of 2% pentobarbital sodium (40 mg/kg), the abdominal skin was disinfected with 75% ethyl alcohol, and enterocelia was opened using a cross-shaped incision to expose heart and postcava. Subsequently, no. 18 trochar was connected to an infusion flask and the perfusate was injected into ventriculus sinister, blood was released from postcava, hepatic perfusion was conducted at a flow velocity of 10 ml/min until the liver turned earthy yellow, followed by perfusion with enzyme perfusate for 15-20 min until the liver became dark brown.

The liver was then washed with normal saline, enveloped and connective tissues were removed and sectioned. The tissue were digested in solution at 37˚C for 20 min, followed by centrifugation at 6 x g for 20 min and the cell suspension was filtered through 200-mesh steel mesh to collect filtrate in two 50 ml centrifugal tubes. The cell suspension was centrifuged at 340 x g for 5 min and the supernatant was discarded. D-Hanks solution was used for re-suspension and deposition, and then centrifuged at 50 x g for 2 min, prior to depositing hepatic cells, and the supernatant was removed to conduct gradient separation. The lymphocyte separation medium was used to pave the gradient, and fully digested single-cell suspension was added to the upper layer and centrifuged at 1,400 x g for 20 min (25˚C). Horizontal projection centrifugation method was used to separate the cells, a small amount of white liquid was carefully adsorbed in the middle layer of HSC. DMEM was centrifuged at 340 x g for 5 min, and washed twice, prior to inoculation of $1x10^6$ cells in 50 ml plastic culture flask under static culture for 24 h. After 72 h, the culture was replaced by DMEM culture solution containing 10% FCS, and the culture solution was replaced once every three days. Trypan blue staining was used to calculate the survival rate of cells, with HSC survival rates of mice in the two groups reaching >90%.

Real-time PCR. Expression of miR-10a in the control and hepatic fibrosis groups were measured using RT-PCR. The TRIzol reagent was used to extract total RNA in the two groups. A SYBR Premix ExTaq fluorescent quantitative PCR kit and LightCycler instrument were used to conduct the operation and analysis.

Inverse transcriptional primer sequence of miR-10a: 5'-GTCGTATC-CAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACACAAA-3'; quantitative upstream primer sequence of miR-10a: 5'-ACGTACCCTGTAGATCCG-3' and downstream sequence: 5'-GTG-CAGGGTCCGAGGT-3'. Inverse transcriptional primer sequence of U6: 5'-CGCTCAC GAATTTGCGTGTCAT-3'; quantitative upstream primer sequence of U6: 5'-CTCGCTTCGGCAGCACA-3' and downstream sequence: 5'-GTG-CAGGGTCCGAGG-3'. DNA amplification was conducted at 94˚C for 15 sec, 94˚C for 20 sec, 55˚C for 10 sec, 72˚C for 10 sec for total of 40 cycles, and final extension at 72˚C for 10 min. The Ct value of the tested sample with PCR was quantified and the U6Ct value was subtracted in the corresponding sample, thereby obtaining the ΔCt value. The expression quantity of miR-10a in hepatic fibroblasts was calculated using $2^{-\Delta\Delta Ct}$, and that in hepatic fibrosis tissue samples was calculated by $\log_2$ transformed, i.e., $\log_2 2^{-\Delta\Delta Ct}$.

Cell culture and transfection of miR-10a mimics. DMEM culture solution containing 10% FCS was used to subculture mouse hepatic fibroblasts in a 5% CO_2 saturated humidity incubator at 37˚C. Fibroblasts in logarithmic growth were inoculated in a 12-well culture plate ($3x10^5$ cells/well), when the degree of cell growth was at 50%. The cells were divided into the miR-10a mimics transfection and control groups. Opti-MEM culture medium containing miR-10a mimics and contrast miRNA, respectively, was used to transfect cells. Subsequently, interferon was added to improve transfection efficiency. Final concentrations of the miR-10a mimics and contrast miRNA in each well during transfection were 20 nmol/l, with interferin at 4 μl, and transfection lasting 72 h and repeated three times.

Cell Counting Kit-8 (CCK-8). The CCK-8 kit was used to test the hepatic fibroblast proliferation capacity according to the protocol. Following transfection of hepatic fibroblasts with miR-10a mimics or contrast to miRNA for 72 h, the cells in the two groups were inoculated into a 96-well culture plate (100 μl/well) at a density of $2x10^4$ cells/well, and three parallel wells were established. When the cells were adhered to the walls after 3-4 h, 100 μl RPMI-1640 culture solution and 10 μl CCK-8 was added, and placed in a 5% CO_2 incubator at 37˚C to be cultured for 2 h. Subsequently, the microplate reader was used to test the optical density (OD) value, and this step was repeated three times.

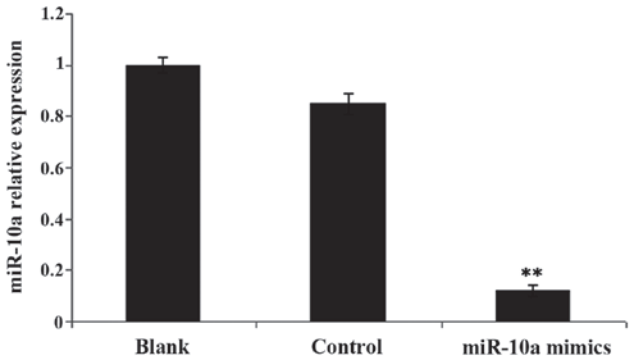

Figure 1. MicroRNA (miR)-10a was low-expressed in hepatic fibrosis cell transfected with miR-10a mimics (**compared with control, P<0.05).

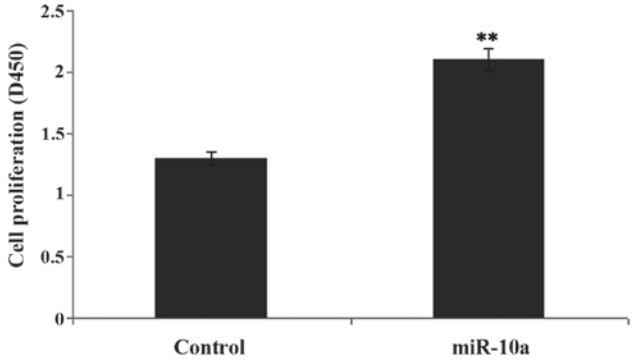

Figure 2. MicroRNA (miR)-10a low-expression promoted the proliferation of hepatic fibroblasts (**compared with the control group, P<0.05).

Western blotting. Following the transfection of hepatic fibroblast for 72 h, the cells in the transfection and control groups were collected and divided using cell lysis solution. The BCA method was used to test the protein concentration, 50 μg protein was taken and separated by 8% SDS-PAGE and transferred to a PVDF membrane at room temperature for 1 h. The primary antibodies, TGF-β1 antibody (1:2,000), (Abcam, Cambridge, MA, USA, catalog no.: ab92486) Smad7 antibody (1:2,000) (Santa Cruz Biotechnology, CA, USA, catalog no.: sc-11392) and β-actin antibody (1:1,000) (Santa Cruz Biotechnology, catalog no.: sc-7210) were added and incubated overnight at 4˚C. The secondary antibody [polyclonal goat-anti-rabbit-HRP (Santa Cruz Biotechnology, catalog no.: sc-2030)] was added with peroxidase after membrane washing and incubated at room temperature for 1 h. ECL was then used to develop it after membrane washing. GeneGnome (Fremont, CA, USA) was used to collect images and ImageJ software (Bethesda, MD, USA) was used for quantification of bands intensity for three replicates.

Statistical analysis. SPSS 18.0 statistical software (Chicago, IL, USA) was used for data analysis. Data were presented as mean ± standard deviation. Matched or non-matched t-test analysis was used for comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-10a is highly expressed in mouse hepatic fibrotic tissues. RT-PCR data showed that miR-10a in fibrotic tissues was significantly higher than that in the control group (-7.84±1.38 vs. -9.97±1.59, P<0.05).

Hepatic fibroblasts had a high expression in miR-10a following transfection of miR-10a mimics. miR-10a expression quantity in cells in the transfection group was approximately 1/16 of that in the control group (Fig. 1).

miR-10a high expression promotes proliferation of hepatic fibroblasts. To examine the effect of a high expression of miR-10a on the proliferation of hepatic fibroblasts, CCK-8 was used to test the proliferation levels in the transfection and control groups. Compared to the control group, proliferation of hepatic fibroblasts with a high expression of miR-10a was significantly increased (P<0.05) (Fig. 2).

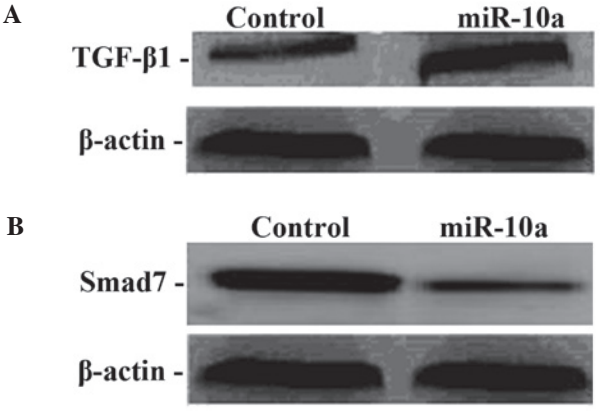

Figure 3. MicroRNA (miR)-10a low-expression significantly increased the expression of transforming growth factor (TGF)-βl (A) and decreased the expression of Smad7 (B) in hepatic fibroblasts.

High miR-10a expression regulates the TGF-βl/Smads signal transduction pathway. To examine the molecular mechanism of miR-10a in the genesis and development of hepatic fibrosis, the protein expression of TGF-βl and Smad7 was determined in hepatic fibroblasts after miR-10a mimics were transfected. Compared to the control group, TGF-βl protein expression in the transfection group was significantly increased, indicating that miR-10a upregulated TGF-βl expression while Smad7 protein expression in the transfection group significantly was decreased, indicating that miR-10a downregulated Smad7 expression (Fig. 3).

Discussion

Previous findings have shown that TGF-βl and its Smad signal transduction pathway are closely associated with the genesis and development of hepatic fibrosis (11,12), whereby TGF-βl activates hepatic fibroblasts and is the strongest hepatic fibrosis accelerator (13). Smad protein is a key active substrate of the TGF-β family receptor kinase. According to difference in structure and function, it is divided into three types, Smad7 as an inhibitory type of Smads mainly

suppresses the TGF-β transduction pathway, Smad7 inhibits Smads phosphorylation by binding activated TGF-β1 receptor, and enters Smad7 cytoplasm, making it impossible for Smads to bind receptors (14). Smad protein constitutes the negative feedback loop in TGF-β signal transduction and exerts its anti-fibrosis effect (14,18).

miRNA is involved in the genesis and development of hepatic fibrosis, although the molecular mechanism of miRNA-regulating hepatic fibrosis remains to be elucidated (15-17). As a member of the miRNA family, miR-10a is involved in the genesis and development of hepatic fibrosis. We found that miR-10a had a high expression in mouse hepatic fibrotic tissues, and this expression was capable of promoting the proliferation of hepatic fibroblasts (19). The results of the present study showed that miR-10a exerted a fibrotic factor-promoting effect in hepatic fibrosis.

We also found that compared to the control group, TGF-βl protein expression in the transfection group significantly increased, indicating that miR-10a downregulated Smad7 expression and exerted a hepatic fibrosis-promoting effect by regulating the TGF-βl/Smads signal transduction pathway, and this provided a potential therapeutic target for the treatment of hepatic fibrosis.

In conclusion, miR-10a expression in mouse hepatic fibrosis tissues increased, whereas a low miR-10a expression promoted the proliferation of hepatic fibroblasts. This proliferative effect was exerted by upregulating TGF-βl expression and downregulating Smad7 expression following the regulation of hte expression of TGF-βl and Smad7 in the TGF-βl/Smads signal transduction pathway. The present study has found a new molecular mechanism for the genesis and development of hepatic fibrosis.

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