

Original Article

Molecular mechanism of miR-154 targeting MMP-9 by regulating Wnt/ β -catenin pathway in liver fibrosis

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Received March 29, 2017; Accepted May 24, 2017; Epub August 1, 2017; Published August 15, 2017

Abstract: This research aimed to study the mechanism of miR-154 targeting MMP-9 by regulating Wnt/ β -catenin pathway the liver fibrosis. Dual luciferase reports were performed to analyze interactions between miR-154 and MMP-9. Pathology of liver fibrosis was observed by HE stains. The expression of β -catenin, Dsh, APC, GSK3 and MMP9 was detected by Western blot and FQ PCR. The Cell proliferation assay indicated miR-154 inhibited cell proliferation. The results of Western blot and FQ PCR show that mRNA and protein levels of β -catenin, Dsh, APC, GSK3, MMP9 decreased significantly, when miR-154 overexpressed in liver fibrosis, while they increased significantly in miR-154 inhibitors group ($P < 0.05$). The luciferase's result displayed the weakened wild-type fluorescents, while no significant differences in mutant fluorescents' strength, compared with the control models. HE's dyeing results the obviously increased in fibrosis, compared inhibitor group with model group. It was concluded that miR-154 inhibited Wnt/ β -catenin's pathway by targeting MMP-9, slowing the process of liver fibrosis.

Keywords: miR-154, MMP9, liver fibrosis, Wnt/ β -catenin pathway

Introduction

Liver fibrosis is a repair process after chronic liver injury. Its main feature is the excessive accumulation of extracellular matrix (extracellular matrix, ECM), in particularly the type I collagen [1]. Excessive deposition of collagen fibers affects the normal liver morphology and function, which will result in cirrhosis or liver cancer if not treated in time. Currently, liver fibrosis is associated with increased morbidity and mortality, becoming one of the main factors threatening human health. MMPs may be activated by some specific protease or organic chemicals such as mercury in vitro, and also it may be activated by tissue and plasma proteases in vivo, and MMPs members can be activated by each other. The MMP-9 can specifically degrade the main components-collagen IV, so that becoming a hot research recently [2]. Many researches showed that MMP9 activated in esophageal squamous cells cancer, squamous cells cancer and laryngeal cancer tissue fibrosis [3-5]. Other studies have shown that MMP9 could activate hepatic stellate cells to promote liver cancer. While in liver fibrosis, the role of MMP9 in its development is reported a little. However, related miRNAs by its upstream

leading to the development of hepatic fibrosis has not been reported.

Mutant miRNA precursors on polymorphism and abnormal methylation affect mature miRNA process. Early literature reports indicate that miR-154 may have a role in the development of liver fibrosis [6-8], and there may be a potential link between miR-154 expression differences and MMP9 by the bioinformatics prediction. We have shown the differential expression in fibrotic liver tissue by the early pre-experiment. MiR-154 is still showed differential expression in liver tissue with no cirrhosis or cirrhosis.

Materials and methods

Reagents

CCI4 was acquired from Sigma (St Louis, MO, USA). Antibody, β -catenin, Dsh, APC, GAPDH, GSK3, and MMP9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

HSC-T6 rat cells were purchased from the study of Chinese Academy of Medical Sciences (Beijing, China). Cells were resuspended in DMEM

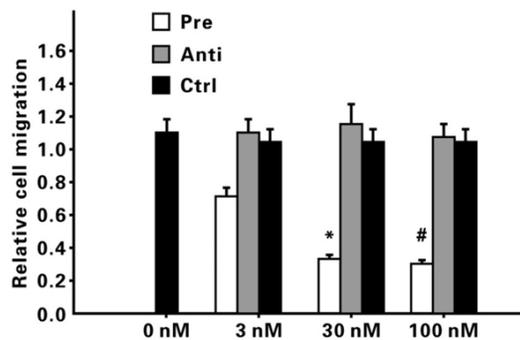


Figure 1. miR-154 in hsc-T6 reduced cell migration. Cells were transfected with different concentrations (0, 3, 30, 100 nM) of pre-miR-154 (pre), anti-miR-154 (anti) and ctrl-miR (ctrl), normalized to non-transfected cells. Data were presented as mean \pm S.E.M., N = 3 and *P < 0.05.

containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA) and maintained in a 37°C with 5% CO₂ [9].

CCl₄ liver injury model

Male adult Sprague-Dawley (180-220 g) rats were provided by CAS Shanghai Life Sciences Research Institute. Liver fibrosis model was established by treatment of 2 ml of CCl₄/olive oil (1:1, v/v)/kg body weight by intraperitoneal injection twice weekly mostly for 6 weeks [10]. Sixty rats were divided into six groups randomly, including olive oil, CCl₄ plus oral phosphate-buffered saline (PBS), CCl₄ plus injected miR-154 inhibitor, CCl₄ plus injected miR-154 mimic, CCl₄ plus injected miR-154 mimics NC; CCl₄ plus injected miR-154 inhibitor NC respectively (GenePharma, China, 80 mg/kg). The protocol was authorized by PLA 107 Hospital. All animals received care in accordance with 'Guide for the Care and Use of Laboratory Animals'. Rats were sacrificed under anesthesia after six weeks and the livers were eliminated for further study. The liver tissues were used for H&E staining by fixation with 10% formalin.

miRNA transfection

The pre-miRTM precursor molecules for miR-154 (pre-miR-154), anti-miRTM inhibitor for miR-154 (anti-miR-154) and an inactive control-miR (ctrl-miR) were gained from Ambion (Austin, TX, USA). HSC-T6 were transfected with 0, 3, 30 or 100 nM of proper miRNAs, as covered before or 100 nM of siRNA with Lipofectamine TM Transfection Agents (Invitrogen), on the basis of manufacturer's guidelines.

Cell proliferation assay

Cells were cultured in a 96-well dish at a density of 1×10^3 cells per well, then cells were transfected with pre-miR-154, anti-miR-154, ctrl-miR as described above. Cell proliferation was confirmed by MTT test on the basis of the instructions of a MTT cell proliferation test kit (Beyotime Biotechnology, Jiangsu, China). The optical density was gauged at 570 nm on a microplate reader (Bio-Rad 550, USA) [11].

Western blot analysis

Tissues and cells were cracked with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM 2-Mercaptoethanol, 2% w/v SDS, 10% glycerol). Total proteins were quantified and separated by SDS-PAGE. Then western blot test was conducted as described previously. The levels of protein were normalized to total GAPDH.

HE stains

The rats were infused transcatheterially with 100 ml of I saline (0.9%). The liver tissues were fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) at 4°C for 24 h, incubated overnight at 4°C in 100 mM sodium phosphate buffer (pH 7.4) containing 30% sucrose; and embedded in optimal cutting temperature compound (Leica, CA, Germany). Cryosections were collected on 3-aminopropyl-trimethoxysilane-coated slides (Sigma-Aldrich). The liver slices were stained with hematoxylin and eosin, and examined by a professor in liver pathology (S.M.) blinded to the type of treatment received by the animals.

Luciferase activity assay

The 3'UTR area of MMP-9 gene was cloned into the pMIR-REPORTTM Luciferase plasmid (Applied Biosystems) to generate pmiR-154 and pmiR-154-Mut vectors. MMP-9 3'UTR for miR-154 forward: 5'-TCGAGTTACGCCGAGTTCAC-3' and reverse: 5'-GTTTCGCCTCTCT AGGGCTC-3'. Transfection was conducted with Lipofectamine 2000 on the basis of the manufacturer's recommendations. pMIR-REPORT β -gal P-con-shRNA was used for transfection normalization. Luciferase values were gauged by Dual-Light System (Applied Biosystems).

Statistical analysis

At least three independent experiments' Data were showed as the mean \pm SD. Statistical

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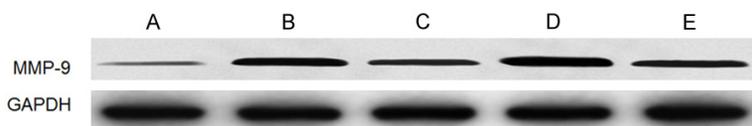


Figure 2. MiR-154 directly targeted MMP-9 (MT3-MMP), the protein expressions of MMP-9 were analyzed by western blotting. GAPDH was used as internal control.

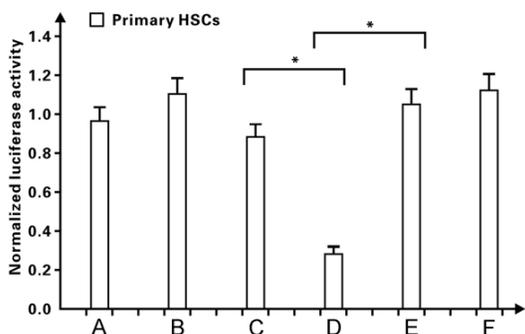


Figure 3. Interaction of miR-154 with the 3'UTR of MMP9. The HSCs were transfected with pmiR (empty vector), pmiR containing miR-154 targeting sequence (pmiR-154) and pMIR with miR-154 mutated targeted sequence (pmiR-154-Mut). The figure showed luciferase activity in cells transfected with pmiR-154 or pmiR-154-Mut. It also showed cotransfection of miR-154 precursor or miR-NC. * $P < 0.05$. A: pmiR + miR-NC. B: pMIR + MIR-154. C: pmiR-154 + MIR-NC. D: pmiR-154 + miR-154. E: pmiR-154-MUT + miR-NC. F: pmiR-154-MUT + miR-154.

analyses was analyzed by using Student's t-test and $P < 0.05$ which was considered significant. All counted analyses were performed with SPSS software (version 19; SPSS, Chicago, IL).

Results

MiR-154 inhibits cell proliferation

Because the increasing miR-154 could improve cell survival, we wondered if it improved cell retention via other mechanisms. The results showed that miR-154 inhibited cell proliferation (**Figure 1**).

MiR-154 reduces MMP-9 levels

We found that MMP-2 and -9 could be guided in HSC-T6, which were important proteases allowing matrix turnover and cell migration. We tested the protein levels of MMP-9 secreted by HSC-T6 on transfection of different miRNAs. Overexpressing miR-154 decreased MMP-9 levels (**Figure 2**). It indicated that miR-154 restricted cell migration by affecting MMP-9

expression. This was authenticated by decreased MMP-9 mRNA levels. So we supposed that MMP-9 might be targeted by miR-154.

MiR-154 was predicted to target MMP-9 and was a larva-ceous inhibitor of MMP-9. So

we examined whether miR-154 could directly target MMP-9. As the expression of MMP-9 was not gauged previously in HSC-T6, we probed and affirmed that MMP-9 was expressed in HSC-T6 as indicated by gene expression upon pre-miR-154 transfection; It suggested that MMP-9 was directly targeted by miR-154. To affirm it, we cloned 1.4 kb of the 3'-UTR of MMP-9 into a luciferase reporter vector. A significant decrease was observed in luciferase expression after cotransfecting miR-154 (**Figure 3**). This inhibition could be totally abolished by cotransfecting with anti-miR-154, indicating that miR-154 directly lowered MMP-9 expression. It was confirmed furtherly by the generation of single gene mutation in the two putative target sites of MMP-9. We could see that the decreased luciferase activity was prevented (**Figure 3**), and the site 1 was the most powerful in regulating MMP-9 expression probably.

Effects of miR-154 on rat liver fibrosis induced by carbon tetrachloride

To confirm the effects of miR-154 on rat liver fibrosis induced by carbon tetrachloride (CCl₄), the degree of rat liver fibrosis was gauged by H&E staining. As indicated by H&E, CCl₄ caused prominent hepatic steatosis, necrosis, and formation of regenerative nodules in rat liver tissues, which was ameliorated by miR-154 mimic (**Figure 4**).

MiR-154 reduces Wnt/ β -catenin pathway activity levels

Previous studies showed that Wnt/ β -catenin pathway is involved in the development of organ fibrosis [12]. To explore whether Wnt/ β -catenin pathway is involved in the effects of Sal B on liver fibrosis, the protein levels of P- β -catenin were detected *in vivo* and *in vitro* after Sal B treatment. The results showed that miR-154 mimic caused a significant increase in the phosphorylation of β -catenin in CCl₄-treated rats, HSC-T6 cells and primary HSCs, miR-154

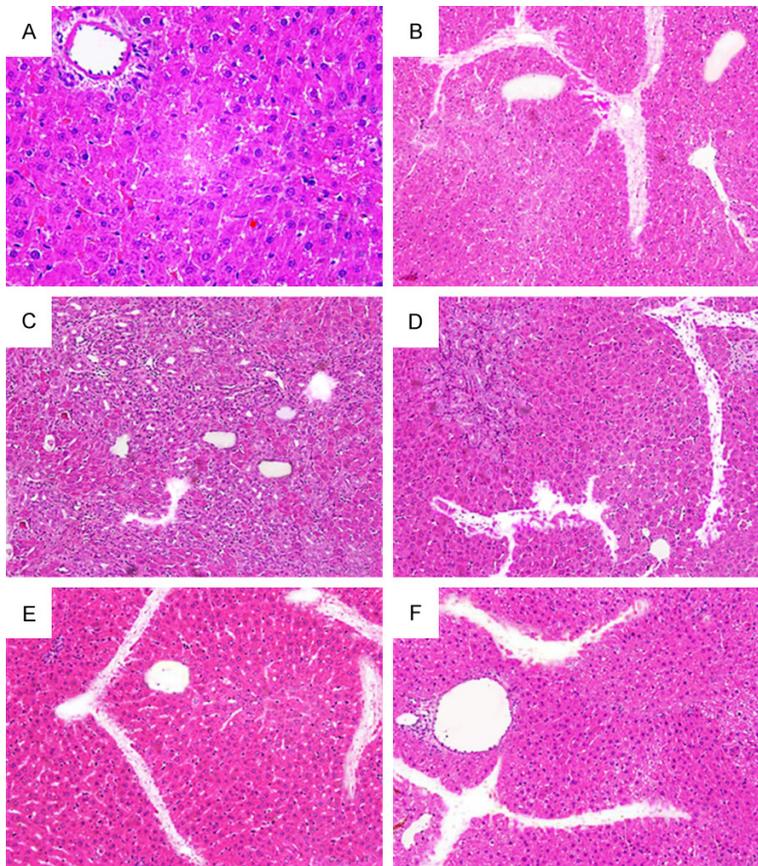


Figure 4. MiR-154 significantly ameliorated CCl4-induced liver fibrosis in rats. H&E staining ($\times 100$) for assessing liver fibrosis. Groups: A: Treated by olive oil. B: Treated by CCl4 plus oral PBS. C: Treated by CCl4 plus injected miR-154 inhibitor. D: Treated by CCl4 plus injected miR-154 mimic. E: Treated by CCl4 plus injected miR-154 mimics NC. F: Treated by CCl4 plus injected miR-154 inhibitor NC.

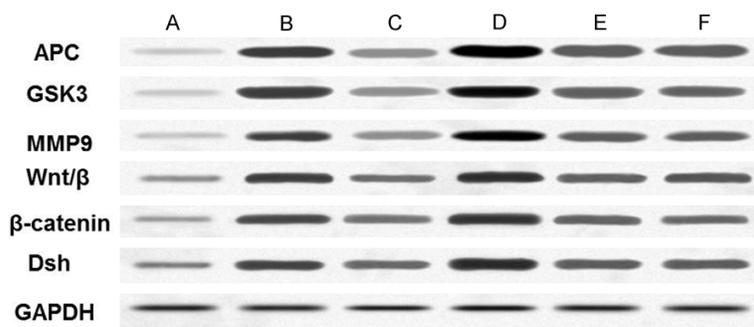


Figure 5. The protein expression of Wnt/beta-catenin signaling pathway. The protein expressions of Wnt, β -catenin, Dsh, APC, GSK3A and MMP-9 were analyzed by western blotting. GAPDH was used as internal control. A: Control; B: CCL4; C: Mimic; D: Inhibitor; E: Mimic control; F: Inhibitor control. The mRNA expression of Wnt/beta-catenin signaling pathway.

mimic treatment also resulted in a reduced TCF activity in HSC-T6 and primary HSCs. To further study whether the suppression of Wnt signaling

caused by Sal B is associated with the increased expressions of Wnt signaling inhibitors, the mRNA expressions of Wnt signaling including Wnt/ β , β -catenin, Dsh, APC, GSK3, MMP9 were detected in CCl4-treated rats, HSC-T6 cells and primary HSCs after Sal B treatment. Western blot analysis further confirmed that miR-154 mimic caused a decrease in Wnt/ β , β -catenin, Dsh, APC, GSK3, MMP9 protein *in vivo* and *in vitro* (Figure 5).

Discussion

Liver fibrosis is a kind of pathological process caused by liver connective tissue dysplasia resulted from a variety of pathogenic factors which cause diffuse intrahepatic excessive precipitation of extracellular matrix [13, 14]. Many chronic liver diseases could induce liver fibrosis. The liver fibrosis is the development center link of a variety of chronic diseases. Inhibition of fibrosis toward cirrhosis and liver cancer prevention is crucial. HSC is the key cell of liver fibrosis process, which promotes differentiation when activated, excess expression in the liver extracellular matrix lately, and ultimately lead to liver fibrosis. Thus suppression of HSC activation and proliferation is an effective treatment on liver fibrosis. Previous studies showed that miR-154 was upregulated in liver fibrosis tissue [15, 16].

It was proposed that the typical Wnt/beta-catenin signaling pathway was one of the main signal transduction pathways of HSC apoptosis in recent years [17]. When the pathway was inactivated, beta-catenin showed the phosphorylation state in

the cytoplasm. While the pathway was activated, beta-catenin was dephosphorylated and translocated to the nucleus, bind to T-cell factor/lymphoid enhancer factor. Target genes induced by Wnt regulation Transcription, promoted the occurrence of liver fibrosis. Some studies have reported that inhibition of Wnt pathway could effectively promote the apoptosis of HSC, and inhibited the occurrence of liver fibrosis [18].

In recent years, microRNA has been found to be involved in the pathological process of many diseases and is a potential therapeutic target in candidate diagnostic marker for disease [2]. It can regulate the expression of genes, and affect the cell differentiation, proliferation, apoptosis, individual growth and development, and so on [16]. A study found that the expression of miR-154 in pulmonary fibrosis cell increased significantly and miR-154 boosted proliferation of cells in pulmonary fibrosis through activation of Wnt/beta-catenin signaling pathway to promote the occurrence of pulmonary fibrosis [19]. Previous studies found that the Wnt/beta catenin signaling pathway was activated in rat hepatic stellate cells, indicated that miR-154 and Wnt/beta catenin signaling pathway may also play an important role in the occurrence and development of hepatic fibrosis. But the miR-154 and Wnt/beta-catenin interactions have not been reported. The results showed that miR-154 targeted MMP-9 to regulate the Wnt/beta-catenin pathway, which defined that miR-154 regulated Wnt/beta-catenin to inhibit the occurrence of liver fibrosis [20-22]. But the role of MMP-9 on the other pathways have not been identified, to lay a theoretical basis for the follow-up study of the effect of miR-154 on liver fibrosis, we need further study.

Disclosure of conflict of interest

None.

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