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Type
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Keywords
simvastatin, IL-10, cirrhosis, miR-34a

Abstract
Introduction
Simvastatin (SIM) treatment has been found to be able to reduce the expression of miR-34a, and we found that interleukin-10 (IL-10) is a potential target gene of miR-34a by searching the online microRNA (miRNA) database. Furthermore, it has been shown that IL10 up-regulation could halt the progression of cirrhosis. The objective of this study was to explore the underlying mechanism of Simvastatin/miR-34a/IL-10 involved in HBV associated cirrhosis.

Material and methods
Real-time PCR, western-blot analysis, immunohistochemistry, computational analysis, luciferase assay was carried out to explore the underlying mechanism of miR-34a involved in HBV associated cirrhosis.

Results
SIM treatment dose-dependently decreased the levels of miR-34a while increasing the levels of IL-10 mRNA and protein. Levels of IL-10 mRNA and protein were remarkably decreased, while miR-34a mRNA level and active caspase-3 protein level was apparently increased in Cirrhosis group compared with sham group. Accordingly, SIM treatment obstructed the dysregulated miR-34a expression and IL-10 expression in cirrhosis animals. By performing computational analysis, we identified that a complementary binding site of miR-34a was located in IL-10 3’ untranslated region (3’UTR), and miR-34a reduced luciferase activity of wild-type IL-10 3’UTR.

Conclusions
Our data also suggested that SIM may become a new therapeutic strategy for HBV-associated cirrhosis via targeting the miR-34a/IL-10 axis.
Administration of simvastatin halts progression of cirrhosis via up-regulating expression of miR-34a and interleukin-10 in rats

Hui Yang¹, Xiao-rong Zhou²*, Yong-hua Wang¹, Yan Cheng¹, Hong-li Zhao¹, Lu Qiao¹

1. Department of Gastroenterology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710004, P.R. China

2. Geriatric Surgery, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710004, P.R. China

*Corresponding author: Dr. Xiao-rong Zhou

Geriatric Surgery, The Second Affiliated Hospital of Xi'an Jiaotong University, No. 157, Xiwu Road, Xincheng District, Xi'an, Shaanxi, P.R. China

Email: surgeondocx@yeah.net

Tel: +86-029-87679387

Running title: Simvastatin halt cirrhosis via miR-34a/IL-10
Abstract

Background: Simvastatin (SIM) treatment has been found to be able to reduce the expression of miR-34a, and we found that interleukin-10 (IL-10) is a potential target gene of miR-34a by searching the online microRNA (miRNA) database. Furthermore, it has been shown that IL10 up-regulation could halt the progression of cirrhosis. The objective of this study was to explore the underlying mechanism of Simvastatin/miR-34a/IL-10 involved in HBV associated cirrhosis. Method: Real-time PCR, western-blot analysis, immunohistochemistry, computational analysis, luciferase assay was carried out to explore the underlying mechanism of miR-34a involved in HBV associated cirrhosis. Results: SIM treatment dose-dependently decreased the levels of miR-34a while increasing the levels of IL-10 mRNA and protein. Levels of IL-10 mRNA and protein were remarkably decreased, while miR-34a mRNA level and active caspase-3 protein level was apparently increased in Cirrhosis group compared with sham group. Accordingly, SIM treatment obstructed the dysregulated miR-34a expression and IL-10 expression in cirrhosis animals. By performing computational analysis, we identified that a complementary binding site of miR-34a was located in IL-10 3’ untranslated region (3’UTR), and miR-34a reduced luciferase activity of wild-type IL-10 3’UTR. Conclusion: Our data also suggested that SIM may become a new therapeutic strategy for HBV-associated cirrhosis via targeting the miR-34a/IL-10 axis. Key word: Simvastatin, cirrhosis, miR-34a, IL-10

Introduction

Liver cirrhosis has become a significant health burden worldwide. Based on the data released by a 2010 study in Global Burden of Disease, liver cirrhosis has resulted in 31 million Disability Adjusted Life Years (DALYs) and 1 million fatalities, accounting for 1.2% of total DALYs and 2% of today deaths worldwide [1]. Although extensive efforts have been made to explore the molecular mechanism underlying the development of cirrhosis, few therapeutic agent has been validated in the treatment of the disease [2,3].
Interleukin-10 (IL-10) has been reported to play an inhibitory role in the development of cirrhosis by decreasing pro-inflammatory responses and regulating hepatic fibrogenesis [4]. In addition, IL-10 was found to exert a direct effect on the synthesis of collagenases and collagen, and hence was involved in the regulation of extracellular matrix (ECM) remodeling [5]. Furthermore, the data from an earlier study showed that IL-10 may be critical to prevent the hepatic fibrogenesis induced by carbon tetrachloride (CCl₄) [6]. Moreover, it has been implicated that the IL-10/IL-10 receptor axis could block the transcription and protein synthesis of matrix metalloproteinase-2 (MMP-2) in immortalized primary human prostate cell strains derived from prostate cancer [7].

As a type of short (18–24 nucleotides in length) and evolutionarily conserved non-coding RNAs, microRNAs (miRNAs) can regulate gene expression at a post-transcriptional level [8]. Studies have demonstrated the involvement of miRNAs in a wide range of biological processes, such as cell differentiation, development and apoptosis [10].

A recent study has demonstrated the involvement of several miRNAs in medical condition of liver including cirrhosis [10]. In addition, it was also reported that HCC could be differentiated from cirrhosis by measuring the profile of microRNAs (miRNAs) in the plasma or serum of the patients and the diagnosis and treatment of cirrhosis in an early stage could decrease the incidence of HCC [14, 15]. Furthermore, Chen et al. used miR-181b and miR-106b circulating in the plasma as biomarkers to achieve early diagnosis of liver cirrhosis with an area under curve (AUC) value of 0.7~0.8 [14]. In another study, up-regulated expression of miR-885-5p was found in the serum of patients suffering from liver cirrhosis (LC) and hepatitis B (HBV). Subsequently, miR-885-5p was used as a candidate biomarker for the diagnosis of cirrhosis [15]. Researchers have found that statins, also known as HMG-CoA reductase inhibitors, are a class of lipid-lowering medications. Statins act to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, which is required for cholesterol synthesis. Statins include rosuvastatin, atorvastatin and pitavastatin, and it promotes the progression of non-alcoholic fatty liver disease (NAFLD) by improving the conditions of hepatitis, fibrosis and hepatic steatosis [20, 21]. In addition, simvastatin was found to decrease the abnormally high
level of liver enzymes and to abolish hepatic inflammation, thus stabilizing or reversing the progress of fibrosis by suppressing the proliferation of HSC [18-21]. Simvastatin treatment has been found to be able to reduce the expression of miR-34a, and we found that IL-10 is a potential target gene of miR-34a by searching the online miRNA database. Furthermore, it has been shown that IL-10 up-regulation could halt the progression of cirrhosis in mouse model [22]. Based on the evidence listed above, we tested the regulatory relationship between simvastatin, miR-34a and IL-10, and also investigated the anti-fibrotic effect of simvastatin in rats as well as its potential signaling pathway.

Materials and Methods

Animal

This is an experimental study which has done on the 36 adult female Wistar rats free of specific-pathogen. The rats were obtained from Institutional Animal Center and weighted 250 ± 20 g. The rats were maintained at 25 ± 2°C along with a schedule of 12-hour light/dark constantly for 2 weeks to acclimatize. All rats were allowed to water and food. Institutional Ethics Committee had already approved this project. Then equal volume olive oil was utilized to dissolve CCl₄, 1 mL/kg body weight CCl₄ was intraperitoneally inject into 24 female rats to induce cirrhosis twice a week, the injection was lasted for 6 weeks. Only olive oil was intraperitoneally injected into 6 female rats to generate normal controls as sham group. Histopathological examination was performed to assess cirrhosis of liver samples. 6 weeks after injection, 24 female rats with cirrhosis were divided into two group: 12 rats with cirrhosis received no treatment as cirrhosis group, other 12 rats with cirrhosis received simvastatin treatment as cirrhosis + simvastain group. 10 mg/kg body weight simvastatin dissolved in 0.5 % solution of xanthan gum was utilized to treat rats orally every day for 8 weeks. Peripheral blood samples were taken from each rat for future study.

RNA isolation and real-time PCR
SV Total RNA Isolation system (Promega, Madison, WI, USA) was utilized to extract total RNA from HepG2 or LO2 cells following instruction indicated by supplier. Total RNA content was examined using spectrophotometrical analysis at 260 nm. RT-PCR kit (Stratagene USA) was utilized to perform RT-PCR in order to reverse transcribe RNA into IL-10 cDNA with a mixture containing 10 μL RNA, 3 μL random primers, RNA primer mixture, 1μL 10 mM deoxynucleotide triphosphates (dNTPs), 1μL RNase inhibitor, 1μL moloneymurine leukemia virus (MMLV)-RT enzyme and 10μL diethylpyrocarbonate (DEPC)-treated water. And the reaction was carried out as follow: 37°C for 60 min, then 95°C for 10 min, and followed by cooling at 4 °C. Quantitative real-time RT-PCR was carried to determine the expression of miR-34a using SYBR Premix Ex TaqTM II (Takara, Dalian, China) following supplier’s guideline. Real-time RT-PCR was performed to determine IL-10 level using standard SYBR Green RT-PCR Kit (Takara, Otsu, Japan) in accordance with supplier’s protocol. Small RNA U6 and GAPDH were served as internal controls for the normalization of miR-34a and IL-10 mRNA respectively. ABI 7500 Software 2.04 from Applied Biosystems (Foster City, CA, USA) with 2^-△△CT method was utilized to calculate the relative expression of miR-34a and IL-10 mRNA normalized to expressions of U6 and GAPDH. All experiments were repeated in triplicate.

Cell culture and transfection

HepG2 or LO2 cells were purchased from Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and DMEM (Dulbecco’s modified Eagle’s medium) (GIBCO, Carlsbad, CA) supplemented with 10% FBS (fetal bovine serum) (GIBCO, Carlsbad, CA), 100 U/mL penicillin and 100 mg/mL streptomycin sulfate was utilized to culture cells at 37°C with a atmosphere with 5% CO₂. 50 nM of miR-34 mimic and its negative control were transfected into HepG2 or LO2 cells using Lipofectamine 2000 (Invitrogen, CA, US) after cells reached 80% confluence. Meanwhile when the cells reached 80% confluence, 1 or 5um simvastatin was utilized to treated HepG2 or LO2 cells for 12 hours. Three independent experiments were carried out.

Cell proliferation assay
HepG2 or LO2 cells were purchased from ATCC and cultured into 12-well plates for 12 hours, and then cells transfected with miR-34 mimic or miRNA mimic negative control, and incubated for additional 24 hours to 72 hours, then 10μL 5 mg/mL MTT was added into each well, and incubated at 37°C for 3 hours with 5% CO₂. Then MTT solution were removed, and 100μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added into each well to dissolve the crystals. A spectrophotometric analysis (BioTek, Grand Island, NY, USA) was utilized to measure cell proliferation at 490 nm. Each test was run in triplicate.

**Luciferase assay**

PCR was carried out to amplify full fragment wild-type IL-10 3’UTR with putative binding site of miR-34a, above PCR products were then subcloned into p-GL3-control vector (Ambion, Austin, TX, USA) to generate Wt- IL-10-3’UTR. Quick Change Site-Directed Mutagenesis Kit (Agilent, Roseville City, CA) was utilized to obtain mutant IL-10 3’UTR, and also subcloned into same site of pGL3-control vector (Ambion, Austin, TX, USA) to generate Mut- IL-10-3’UTR located downstream of luciferase gene. Then HepG2 or LO2 cells were maintained into 24-well plate, Lipofectamine 2000 (Invitrogen, CA, US) was utilized to co-transfect constructs contained wild-type or mutant IL-10 3’UTR and miR-34a or miR-NC into HepG2 or LO2 cells based on manufacturer’s guideline. 48 hours after transfection, Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was utilized to measure luciferase activity of Firefly luciferase and Renilla luciferase. Three independent experiments were carried out.

**Western blot analysis**

48 hours after transfection with miR-34a mimic, ice-cold PBS was utilized to wash the HepG2 or LO2 cells three times, and radioimmuno precipitation assay (Keygen, Nanjing, China) buffer supplemented with 1mM phenylmethanesulfonylfluoride fluoride (Keygen) was utilized to lyse the cell in accordance with supplier’s description. The lysates were subjected to centrifugation at 12000×g at 4°C for 15 min. BCA protein assay kit (TaKaRa, Japan) was utilized to examine concentration of protein based on
manufacturer’s instruction. 8-12% SDS-PAGE (Bio-Rad Laboratories, Hertfordshire, UK) was utilized to separate total protein, and then transferred to PVDF (polyvinylidene difluoride) membrane (Immobilon-P; Millipore, Billerica, MA, USA) for 90 min at 120V.

5% no-fat milk was utilized to block membrane for 120 min at room temperature. The primary antibodies against IL-10 (1:6,000; Biorbyt Limited, Cambridge, UK) or anti-β-actin (1:10,000; Biorbyt Limited, Cambridge, UK) was utilized to treat the membrane at 4°C overnight, and TBST (Tris buffered saline with 1% Tween) was utilized to wash the membrane three times, and HRP (horseradish peroxidase)-labeled secondary antibody (1:15,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was utilized to treat the membrane at room temperature for 120 min. Enhanced chemiluminescence (Tanon, Shanghai, China) was utilized to visualize specific bands. All tests were repeated in triplicate.

**Apoptosis analysis**

48 hours following transfection, the cells were collected, and PBS was utilized to wash the cells. FITC-Annexin V/propidium iodide Apoptosis Detection Kit (BestBio, Shanghai, China) was utilized to treat HepG2 and LO2 cells based on standard guideline indicated by supplier. The flow cytometry (BD FACSCanto II, BD Biosciences, San Jose, USA) was utilized to analyze the data immediately. All reactions was repeated at least three times.

**Masson staining**

Fontana-Masson kit (Abcam, Cambridge, MA, USA) was utilized to perform Masson staining according to standard protocol. In brief, liver tissues embedded with paraffin were cut into 4 μM thickness sections, hexahydro toluene and gradient ethanol were utilized to dewax sections, then rehydrated. Weigert’s hematoxylin containing ferric chloride in diluted hydrochloric acid, potassium ferricyanide solution alkaliized by sodium borate and hematoxylin in 95% ethanol was used to stain the nuclei of cells. Then plasma stain supplemented with acid fuchsin, Xyldine Ponceau, glacial acetic acid, and distilled water was utilized to maintain tissue samples. Solution containing
phosphomolybdic acid in distilled water was subsequently utilized to treat tissue samples. Fibre stain with Light Green SF yellowish was used to stain collagen finally.

**TUNEL**

TUNEL assay was utilized to examine apoptosis of sections. Briefly, 50 μL TUNEL reaction buffer (Roche Applied Science, Bael, Swiss) was utilized to treat sections in the darkness at 37°C for 1 hour in a humidified atmosphere. DAPI was utilized to stain cell nuclei by maintaining with the sections at RT in the darkness for 5 min. Then flow cytometry was utilized to determine number of positive cells. The ratio was calculated as number of TUNEL positive cells / total cells.

**Statistical analysis**

All data were shown as mean ± SD (standard deviation). SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA) was utilized to perform statistical analysis. Independent t-test was utilized to analyze comparisons of continuous data, χ² test was utilized to analyze categorical data. P value less than 0.05 was considered to be statistically significant.

**Results**

**IL-10 is a candidate gene of miR-34a**

Bioinformatics algorithms including TargetScan were utilized to predict miR-34a target gene. Based on the results of algorithms above, we predicted IL-10 might be a possible target gene of miR-34a with a complementary seed region of miR-34a (Fig. 1A). To further confirm IL-10 is a candidate gene of miR-34a, we then conducted luciferase assay, and sub-cloned wild or mutant IL-10 3’UTR into luciferase reporter which located direct downstream of luciferase gene. Then HepG2 and LO2 cells co-transfected with luciferase reporter carried wild or mutant IL-10 3’UTR and different dose of miR-34a. Luciferase activity of wild-type IL-10 3’UTR in HepG2 (Fig. 1B) and LO2 (Fig. 1D) cells showed a stepwise decline as the concentration of miR-34a mimics increased when compared with the negative controls, and miR-34a had no effect on luciferase activity of
mutant IL-10 3’UTR in HepG2 (Fig. 1C) and LO2 (Fig. 1E) cells, indicating that miR-34a directly targeted IL-10.

Effect of simvastatin on transcription activity of miR-34a promoter

To further confirm whether simvastatin affected transcription activity of miR-34a promoter, we then conducted luciferase assay, and sub-cloned miR-34a promoter into luciferase reporter which located direct upstream of luciferase gene (Fig. 2A), then different dose of simvastatin was utilized to treated HepG2 and HepG2 cells transfected with constructs containing miR-34a promoter. As shown in Fig. 2, luciferase activity of miR-34a promoter in HepG2 (Fig. 2B) and LO2 (Fig. 2C) cells showed a stepwise decline as the concentration of simvastatin increased when compared with the negative controls, suggesting that simvastatin inhibited transcription activity of miR-34a promoter.

Effect of simvastatin on miR-34a and IL-10 levels

Real-time PCR and western-blot analysis were utilized to examine levels of miR-34a and IL-10 in HepG2 and LO2 cells treated with different dose of simvastatin. As shown in Fig. 3, miR-34a level in HepG2 (Fig.3A) and LO2 (Fig. 3E) cells treated with simvastatin was reduced compared with control under a dose-dependent manner, while simvastatin dose-dependently enhanced IL-10 mRNA (Fig. 3B and 3F) and protein (Fig. 3C, 3D, 3G, 3H) expressions in HepG2 (Fig. 3A-D) and LO2 (Fig. 3E-H) cells compared with control.

MiR-34a and IL-10 varied among different groups

All animals were divided into three groups: sham, cirrhosis and cirrhosis treated with simvastatin. And miR-34a and IL-10 among above three groups using real-time PCR and western-blot analysis. As shown in Fig. 4A, animals diagnosed with cirrhosis evidently increased miR-34a compared with sham group, while administration of simvastatin partially restored expression of miR-34a. IL-10 mRNA (Fig. 4B) and protein (Fig. 4C) levels in cirrhosis group were much lower than cirrhosis + simvastatin group, both of them were much lower than sham group. Also, animals diagnosed with cirrhosis
evidently increased caspase-3 level compared with sham group, while administration of simvastatin partially restored expression of caspase-3. Masson staining was performed to detect degree of cirrhosis among sham, cirrhosis and cirrhosis+ simvastatin groups. As shown in Fig. 4E, degree of cirrhosis in cirrhosis group were much higher than cirrhosis + simvastatin group, both of them were much higher than sham group.

Differential apoptosis among various groups

TUNEL assay was performed to determine apoptosis among sham, cirrhosis and cirrhosis+ simvastatin groups. As shown in Fig. 4F, apoptosis in cirrhosis group was much higher than cirrhosis + simvastatin group, both of them were much higher than sham group.

Discussion

Using rodent cirrhosis models, several preclinical trials have demonstrated the potential advantages of statins to treat portal hypertension [23]. Furthermore, a pilot study on cirrhosis patients suggested that one single dose of simvastatin given by oral administration could result in a sharp decline in vascular resistance of the liver [24]. Subsequently, a randomized and placebo-controlled multicenter study demonstrated that a one-month treatment with simvastatin led to a decreased portal pressure and improved clearance of indocyanine green, suggesting the improvement in liver functions [25]. Overall, these data indicated that, in the cirrhosis patients suffering from acute variceal bleeding, simvastatin may ameliorate the prognosis of these patients by influencing the two key factors involved, i.e., liver functions and portal pressure. In particular, a recent clinical trial also obtained similar data demonstrating that simvastatin acted as a liver-selective and potent vasodilator, since the systemic vascular resistance and average arterial pressure remained stable after the prolonged administration of simvastatin [25]. In this study, we detected levels of miR-34a, IL-10 mRNA and protein level among the rat model groups using real-time PCR and western-blot analysis, and validated that miR-34a level in cirrhosis group was much higher than cirrhosis + simvastatin group, both of them were much higher than sham group.
However, IL-10 expression in cirrhosis group was much lower than cirrhosis + simvastatin group, both of them were much lower than sham group. Moreover, we performed TUNEL assay to detect apoptosis among sham, cirrhosis and cirrhosis + simvastatin groups, and found that apoptosis in cirrhosis group was much higher than cirrhosis + simvastatin group, both of them were much higher than sham group.

A previous study has shown that, via the suppression of miR-34a expression, the application of atorvastatin could benefit endothelial functions by increasing the expression of SIRT1 [21]. Furthermore, during the progression of NAFLD, the levels of apoptosis, acetylated p53 and microRNA-34a in the fibrotic tissues of the liver were all gradually elevated [30]. These results suggested that statins, especially simvastatin, may play a critical role in improving hepatic inflammation, steatosis and fibrosis by regulating the pathways related to microRNA-34a [27]. In human patients or animal models of NAFLD, cirrhosis, alcoholic liver injury and HCC, the expression of miR-34a was elevated and was dependant on the severity of the disease [28]. In addition, miR-34a was found as a direct target of p53. Since sirtuin 1 (SIRT1) acts as the target gene of miR-34a and can suppress p53-dependent apoptosis via the deacetylation of all major sites involved in p53 acetylation, a positive loop of feedback is formed along the SIRT1/miR-34a/p53 signaling pathway, which in turn plays a critical role in regulating the apoptosis and proliferation of cells [29, 30]. It was also shown that, by targeting IL-10, miR-34 exerted an indirect effect on the induction of MDSC [31]. In this study, we carried out computational analysis to predict miR-34a target gene, and identified that IL-10 as a virtual target gene of miR-34a, and luciferase activity of wild-type IL-10 3’UTR was dose-dependently reduced by transfecting with miR-34a. In addition, we investigate effect of simvastatin on transcription activity of miR-34a promoter using luciferase assay, and found that simvastatin inhibited luciferase activity droved by miR-34a promoter under a dose-dependent manner.

As an anti-inflammatory cytokine, interleukin-10 (IL-10) is synthesized by monocytes/macrophages, regulatory T cells and Th2 cells. IL-10 can inhibit the production of cytokines, including IFNγ from T cells as well as TNF-α, IL-1α, IL-1β and IL-6.
from activated macrophages [32]. Located on chromosome 1 (1q31-1q32), the gene encoding IL-10 is about 4.7 kb and includes five exons and four introns [33]. A few studies have investigated the effect of IL-10 gene polymorphisms on the susceptibility to liver cirrhosis, although their conclusions were inconsistent [34]. For example, it was shown that the rs1800896 polymorphism of IL-10 was associated with the elevated risk of liver cirrhosis, particularly in patients suffering from chronic infection of hepatitis B [35]. In this study, we performed real-time PCR and western-blot analysis to detect influence of simvastatin on expressions of miR-34a and IL-10, and revealed that simvastatin decreased miR-34a level under a dose-dependent manner, while enhanced IL-10 expression under a dose-dependent manner.

As an important and pleiotropic cytokine with immunoregulatory features, interleukin 10 (IL-10) is mainly produced in macrophages, although it can also be synthesized in mast cells, monocytes, dendritic cells, B lymphocytes, cytotoxic T cells, as well as in T helper 1 (Th1) cells, Th2 cells and even in human carcinoma cell lines [36]. The activity of IL-10 is regulated by IL-10 receptor (IL-10R), a member of the class II cytokine receptor family. By inhibiting the expression of major histocompatibility complex (MHC) class II and co-stimulatory factors including CD80 (B7.1) and CD86 (B7.2), IL-10 reduces the ability of macrophages and monocytes to present antigens to T cells, thus reducing the expression of tumor necrosis factor alpha (TNF-α), IL-1, IL-6, IL-8, and IL-12. In addition, it was found that IL-10 could prevent the apoptosis of B cells and enhance their proliferation, thus playing a role of immunoglobulin (Ig) class switch.

As a cytokine that reduces pro-inflammatory responses and regulates hepatic fibrogenesis, IL-10 may provide a therapeutic alternative for patients with HCV-related cirrhosis that do not respond to IFN-based therapy [37, 38]. In addition, IL-10 was found to ameliorate fibrosis by suppressing the activity of HSC [39]. Similar results were also obtained using a rat model, in which the administration of exogenous IL-10 reversed CCl₄-induced fibrosis in the liver by decreasing the expression of TIMP-1 and TGF-β1 [37].

Conclusion
In our study, we suggested that administration of simvastatin halts progression of cirrhosis via up-regulating expression of miR-34a and interleukin-10 in rats. In brief, we found that Simvastatin treatment has been found to be able to reduce the expression of miR-34a, and we found that IL-10 is a potential target gene of miR-34a by searching the online miRNA database. Furthermore, we revealed that IL-10 up-regulation could halt the progression of cirrhosis in mouse model.

Conflict of interest

None

Acknowledgements

None.

References


**Figure legends**

**Figure 1**

IL-10 is confirmed as a candidate gene of miR-34a by computational analysis and luciferase assay (WT: wild-type; MUT: mutant; N=3; * P value < 0.05 vs. 0 group).

A: Comparison between miR-34a and wild-type/mutant IL-10 3’UTR.

B: Luciferase activity of wild-type IL-10 3’UTR was inhibited under a dose-dependent manner in HepG2 cells

C: Luciferase activity of wild-type IL-10 3’UTR was dose-dependently decreased under a dose-dependent manner in LO2 cells

D: MiR-34a had no effect on luciferase activity of mutant IL-10 3’UTR in HepG2 cells

E: MiR-34a had no effect on luciferase activity of mutant IL-10 3’UTR in LO2 cells

**Figure 2**

Effect of simvastatin on transcription activity of miR-34a promoter detected by luciferase assay (SIM: simvastatin; N=3; * P value < 0.05 vs. untreated group).

A: MiR-34a promoter was inserted into luciferase reporter which located direct upstream of luciferase gene

B: Luciferase activity droved by miR-34a promoter in HepG2 cells showed a stepwise decline as the concentration of simvastatin increased when compared with the negative controls

C: Luciferase activity droved by miR-34a promoter in LO2 cells showed a stepwise decline as the concentration of simvastatin increased when compared with the negative controls
Figure 3

Simvastatin varied expressions of miR-34a and IL-10 in HepG2 and LO2 cells detected by real-time PCR and Western-blot analysis (SIM: simvastatin; N=3; * P value < 0.05 vs. untreated group).

A: Simvastatin dose-dependently inhibited miR-34a expression

B: IL-10 mRNA level was increased subsequent to treat with simvastatin under a dose-dependent manner

C: IL-10 protein expression was enhanced subsequent to treat with simvastatin under a dose-dependent manner

D: IL-10 protein expression was enhanced subsequent to treat with simvastatin under a dose-dependent manner

E: Simvastatin dose-dependently inhibited miR-34a expression

F: IL-10 mRNA level was increased subsequent to treat with simvastatin under a dose-dependent manner

G: IL-10 protein expression was enhanced subsequent to treat with simvastatin under a dose-dependent manner

H: IL-10 protein expression was enhanced subsequent to treat with simvastatin under a dose-dependent manner

Figure 4

Differential levels of miR-34a and IL-10, degree of cirrhosis and apoptosis status among different groups detected by real-time PRC, Western-blot analysis, Masson staining and TUNEL assay (Sham: sham-operated; N=3; * P value < 0.05 vs. sham group; ** P value < 0.05 vs. Cirrhosis group).

A: MiR-34a level in cirrhosis + simvastatin group was much higher than sham group, which was even higher in cirrhosis group than cirrhosis + simvastatin group
B: IL-10 mRNA in cirrhosis + simvastatin group was much lower than sham group, which was even lower in cirrhosis group than cirrhosis + simvastatin group.

C: IL-10 protein in cirrhosis + simvastatin group was much lower than sham group, which was even lower in cirrhosis group than cirrhosis + simvastatin group.

D: Caspase-3 protein in cirrhosis + simvastatin group was much higher than sham group, which was even higher in cirrhosis group than cirrhosis + simvastatin group.

E: Degree of cirrhosis in cirrhosis group were much higher than cirrhosis + simvastatin group, both of them were much higher than sham group.

F: Apoptosis in cirrhosis group was much higher than cirrhosis + simvastatin group, both of them were much higher than sham group.
IL-10 is confirmed as a candidate gene of miR-34a by computational analysis and luciferase assay (WT: wild-type; MUT: mutant; N=3; * P value < 0.05 vs. 0 group).

A: Comparison between miR-34a and wild-type/mutant IL-10 3'UTR.
B: Luciferase activity of wild-type IL-10 3'UTR was inhibited under a dose-dependent manner in HepG2 cells.
C: Luciferase activity of wild-type IL-10 3'UTR was dose-dependently decreased under a dose-dependent manner in LO2 cells.
D: MiR-34a had no effect on luciferase activity of mutant IL-10 3'UTR in HepG2 cells.
E: MiR-34a had no effect on luciferase activity of mutant IL-10 3’UTR in LO2 cells
Effect of simvastatin on transcription activity of miR-34a promoter detected by luciferase assay (SIM: simvastatin; N=3; * P value < 0.05 vs. untreated group).

A: MiR-34a promoter was inserted into luciferase reporter which located direct upstream of luciferase gene.

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C: Luciferase activity drove by miR-34a promoter in LO2 cells showed a stepwise decline.
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B: IL-10 mRNA in cirrhosis + simvastatin group was much lower than sham group, which was even lower in cirrhosis group than cirrhosis + simvastatin group
C: IL-10 protein in cirrhosis + simvastatin group was much lower than sham group, which was even lower in cirrhosis group than cirrhosis + simvastatin group
D: Caspase-3 protein in cirrhosis + simvastatin group was much higher than sham group, which was even higher in cirrhosis group than cirrhosis + simvastatin group
E: Degree of cirrhosis in cirrhosis group were much higher than cirrhosis + simvastatin group, both of them were much higher than sham group
F: Apoptosis in cirrhosis group was much higher than cirrhosis + simvastatin group, both of them were much higher than sham group.