Administration of simvastatin halts progression of cirrhosis via up-regulating expression of miR-34a and interleukin-10 in rats

Type
Research paper

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 Zhou ²*, Yong-hua Wang ¹, Yan Cheng ¹, Hong-li Zhao ¹, Lu Qiao ¹

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 blocked the transcription and protein synthesis of matrix metalloproteinase-2 (MMP-2) in in non-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 rosvastatin, 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^ΔΔCT 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℃ 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℃ 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 alkalized 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 + simvastain 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 IFNy 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

1. Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, et al.: Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2197-2223.

2. Bosetti C, Levi F, Lucchini F, Zatonski WA, Negri E, La Vecchia C: Worldwide mortality from cirrhosis: an update to 2002. J Hepatol 2007;46:827-839.

3. Rani M, Yang B, Nesbit R: Hepatitis B control by 2012 in the WHO Western Pacific Region: rationale and implications. Bull World Health Organ 2009;87:707-713.

4. Farkas Z, Rashid T, Chen Y, Siddiqui T, Yandrapalli S, Frager S, Aronow W, Bodin R, Maddineni S: The correlation between sarcopenia and post-transjugular intrahepatic portosystemic shunt hepatic encephalopathy: a single-institution review. Archives of Medical Science - Atherosclerotic Diseases. 2019;4(1):89-93.

5. Louis H, Le Moine O, Goldman M, Deviere J: Modulation of liver injury by interleukin-10. Acta Gastroenterol Belg 2003;66:7-14.
Huang YH, Shi MN, Zheng WD, Zhang LJ, Chen ZX, Wang XZ: Therapeutic effect of interleukin-10 on CCl4-induced hepatic fibrosis in rats. World J Gastroenterol 2006;12:1386-1391.

Stearns ME, Wang M, Hu Y, Garcia FU, Rhim J: Interleukin 10 Blocks Matrix Metalloproteinase-2 and Membrane Type 1-Matrix Metalloproteinase Synthesis in Primary Human Prostate Tumor Lines. Clin Can Res 2013; 9(3).

Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-297.

He L, Hannon GJ: MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522-531.

Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, Tang ZY, Wang XW: Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 2008;47:897-907.

Qi P, Cheng SQ, Wang H, Li N, Chen YF, Gao CF: Serum microRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. PLoS One 2011;6:e28486.

Bolondi L, Sofia S, Siringo S, Gaiani S, Casali A, Zironi G, Piscaglia F, Gramantieri L, Zanetti M, Sherman M: Surveillance programme of cirrhotic patients for early diagnosis and treatment of hepatocellular carcinoma: a cost effectiveness analysis. Gut 2001;48:251-259.

Xiong J, Zhang M, Guo X, Pu L, Xiong H, Xiang P, Liu J, Li A: Acute kidney injury in critically ill cirrhotic patients with spontaneous bacterial peritonitis: a comparison of KDIGO and ICA criteria. Arch Med Sci 2020;16(3):569-576.

Chen YJ, Zhu JM, Wu H, Fan J, Zhou J, Hu J, Yu Q, Liu TT, Yang L, Wu CL, Guo XL, Huang XW, Shen XZ: Circulating microRNAs as a Fingerprint for Liver Cirrhosis. PLoS One 2013;8:e66577.
Gui J, Tian Y, Wen X, Zhang W, Zhang P, Gao J, Run W, Tian L, Jia X, Gao Y: Serum microRNA characterization identifies miR-885-5p as a potential marker for detecting liver pathologies. Clin Sci (Lond) 2011;120:183-193.

Miyaki T, Nojiri S, Shinkai N, Kusakabe A, Matsuura K, Iio E, Takahashi S, Yan G, Ikeda K, Joh T: Pitavastatin inhibits hepatic steatosis and fibrosis in non-alcoholic steatohepatitis model rats. Hepatol Res 2011;41:375-385.

Socha M, Pietrzak A, Grywalska E, Pietrzak D, Matosiuk D, Kiciński P, Rolinski J: The effect of statins on psoriasis severity: a meta-analysis of randomized clinical trials. Arch Med Sci 2020;16(1):1-7.

Abel T, Feher J, Dinya E, Eldin MG, Kovacs A: Safety and efficacy of combined ezetimibe/simvastatin treatment and simvastatin monotherapy in patients with non-alcoholic fatty liver disease. Med Sci Monit 2009;15:MS6-11.

Ekstedt M, Franzen LE, Mathiesen UL, Holmqvist M, Bodemar G, Kechagias S: Statins in non-alcoholic fatty liver disease and chronically elevated liver enzymes: a histopathological follow-up study. J Hepatol 2007;47:135-141.

Rombouts K, Kisanga E, Hellemans K, Wielant A, Schuppan D, Geerts A: Effect of HMG-CoA reductase inhibitors on proliferation and protein synthesis by rat hepatic stellate cells. J Hepatol 2003;38:564-572.

Tabuchi T, Satoh M, Itoh T, Nakamura M: MicroRNA-34a regulates the longevity-associated protein SIRT1 in coronary artery disease: effect of statins on SIRT1 and microRNA-34a expression. Clin Sci (Lond) 2012;123:161-171.

Elmasry A, Aladeeb NM, Elkaref A, Aboulfotouh N: Simvastatin exerts antifibrotic effect and potentiates the antischistosomal effects of praziquantel in a murine model: Role of IL10. Biomed Pharmacother 2017;96:215-221.
Abraldes JG, Rodriguez-Vilarrupla A, Graupera M, Zafra C, Garcia-Caldero H, Garcia-Pagan JC, Bosch J: Simvastatin treatment improves liver sinusoidal endothelial dysfunction in CCl4 cirrhotic rats. J Hepatol 2007;46:1040-1046.

Zafra C, Abraldes JG, Turnes J, Berzigotti A, Fernandez M, Garca-Pagan JC, Rodes J, Bosch J: Simvastatin enhances hepatic nitric oxide production and decreases the hepatic vascular tone in patients with cirrhosis. Gastroenterology 2004;126:749-755.

Abraldes JG, Albillos A, Banares R, Turnes J, Gonzalez R, Garcia-Pagan JC, Bosch J: Simvastatin lowers portal pressure in patients with cirrhosis and portal hypertension: a randomized controlled trial. Gastroenterology 2009;136:1651-1658.

Castro RE, Ferreira DM, Afonso MB, Borralho PM, Machado MV, Cortez-Pinto H, Rodrigues CM: miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease. J Hepatol 2013;58:119-125.

Wang W, Zhao C, Zhou J, Zhen Z, Wang Y, Shen C: Simvastatin ameliorates liver fibrosis via mediating nitric oxide synthase in rats with non-alcoholic steatohepatitis-related liver fibrosis. PLoS One 2013;8:e76538.

Meng F, Glaser SS, Francis H, Yang F, Han Y, Stokes A, Staloch D, McCarra J, Liu J, Venter J, Zhao H, Liu X, Francis T, Swendsen S, Liu CG, Tsukamoto H, Alpini G: Epigenetic regulation of miR-34a expression in alcoholic liver injury. Am J Pathol 2012;181:804-817.

He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D, Jackson AL, Linsley PS, Chen C, Lowe SW, Cleary MA, Hannon GJ: A microRNA component of the p53 tumour suppressor network. Nature 2007;447:1130-1134.
Yamakuchi M, Lowenstein CJ: MiR-34, SIRT1 and p53: the feedback loop. Cell Cycle 2009;8:712-715.

Wang X, Chang X, Zhuo G, Sun M, Yin K: Twist and miR-34a are involved in the generation of tumor-educated myeloid-derived suppressor cells. Int J Mol Sci 2013;14:20459-20477.

D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G: Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 1993;178:1041-1048.

Damgaard Sandahl T: Alcoholic hepatitis. Dan Med J 2014;61:B4755.

Liu Y, Yu MC, Zhang AQ, Wang YB, Jiang K, Dong JH: Interleukin-10 gene promoter polymorphism and risk of liver cirrhosis. Genet Mol Res 2015;14:1229-1234.

Yao L, Xing S, Fu X, Song H, Wang Z, Tang J, Zhao Y: Association between interleukin-10 gene promoter polymorphisms and susceptibility to liver cirrhosis. Int J Clin Exp Pathol 2015;8:11680-11684.

Gastl GA, Abrams JS, Nanus DM, Oosterkamp R, Silver J, Liu F, Chen M, Albino AP, Bander NH: Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. Int J Cancer 1993;55:96-101.

Chou WY, Lu CN, Lee TH, Wu CL, Hung KS, Concejero AM, Jawan B, Wang CH: Electroporative interleukin-10 gene transfer ameliorates carbon tetrachloride-induced murine liver fibrosis by MMP and TIMP modulation. Acta Pharmacol Sin 2006;27:469-476.

Nelson DR, Lauwers GY, Lau JY, Davis GL: Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders. Gastroenterology 2000;118:655-660.
Zhang LJ, Zheng WD, Chen YX, Huang YH, Chen ZX, Zhang SJ, Shi MN, Wang XZ: Antifibrotic effects of interleukin-10 on experimental hepatic fibrosis. Hepatogastroenterology 2007;54:2092-2098.

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 PCR, 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: Capase-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.
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
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 drove 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 drove 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: Capase-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.