

Original Article

Expression of miR-467b in atherosclerosis and its effects on TNF- α and MCP-1 in RAW 264.7 macrophages

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Abstract: This study aims to investigate the expression of miR-467b in atherosclerosis (AS) and its effects on the expression of tumor necrosis factor α (TNF- α) and monocyte chemoattractant protein-1 (MCP-1) in RAW 264.7 macrophages. 20 ApoE^{-/-} mice were randomly divided into the control group (n = 10) and atherosclerosis model group (AS model group) (n = 10). The control group received a standard diet, while the mice in AS model group were given a high-fat diet (containing 0.25% cholesterol and 15% cocoa butter) to induce atherosclerotic plaque. Then real-time quantitative PCR (RT-qPCR) was applied to detect the expression of miR-467b in thoracic aorta tissues of ApoE^{-/-} mice. Here we utilized RAW 264.7 macrophages that were transfected with miR-467b mimics or inhibitors to detect the expression of TNF- α and MCP-1 by RT-qPCR, and analyzed the effects of miR-467b on the expression of TNF- α and MCP-1; meanwhile, bioinformatics software was used to predict potential target site for miR-467b. And the results showed the presentation of thoracic aorta atherosclerotic plaques in ApoE^{-/-} mice after receiving high-fat diet. Moreover, the expression of miR-467b in thoracic aorta tissue of AS mice was significantly down-regulated compared to the control group, and the difference was statistically significant (P < 0.05). And the expression of miR-467b in macrophages largely inhibited the level of TNF- α and MCP-1. Meanwhile, bioinformatics software showed that miR-467b was likely to target lipoprotein lipase (LPL). In conclusion, miR-467b expression was down-regulated in AS mice, and miR-467b low-expression can induce the expression of TNF- α and MCP-1 in macrophages by promoting LPL expression.

Keywords: Atherosclerosis (AS), miR-467b, TNF- α , MCP-1

Introduction

Angiocardiopathy, including apoplexy and myocardial infarction, causes over 17 million people to die per year in the world [1]. The main reason of that is occlusive thrombus formed due to the rupture of atherosclerotic plaque. Atherosclerosis is generally regarded as the result of unhealthy diet. Its potential effect factors contain dyslipoproteinemia, hypertension, diabetes, obesity, smoke, sedentary lifestyle [2]. However, the continue investigation shows that AS not only happened in the group with such symptoms above. CT-scan for patient group with healthy lifestyle shows that AS formed not only because of the aggregation of cholesterol, but also due to the participation of chronic vasculitis caused by such aggregation

[3]. TNF- α is an important inflammatory factor which can lead to the dysfunction of blood vessel endothelium and accelerate the forming of AS through breaking the integrity of vascular endothelial cell (VEC) [4, 5]. Another cell factor is MCP-1 which can participate in the expression of VEC, vascular smooth muscle cell (VSMC) and macrophages, as well as induce the hyaline leukocyte to move to vascular wall. The interaction of VSMC and hyaline leukocyte is propitious to the forming of early stage AS. Thus MCP-1 is considered as the main factor of vascular wall inflammation [6, 7].

MicroRNAs (miRNAs) are small non-coding RNAs, which participates in the process of many kinds of disease through its own fine tuning effect. Until now many miRNAs related to AS

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Table 1. MicroRNA RT-PCR and PCR specific primers

Target	Application	Primers
U6 snRNA	RT	5'-AAC GCT TCA CGA ATT TGC GT-3'
	PCR	F: 5'-CTC GCT TCG GCA GCA CA-3' R: 5'-AAC GCT TCA CGA ATT TGC GT-3'
Mmu-miR-467b	RT	5'-GTC GTA TCC AGT GCG TGT CGT GGA GTC GGC AAT TGC ACT GGA TAC GAC CAT ATA C-3'
	PCR	F: 5'-GTA AGT GCC TGC ATG TAT ATG-3' R: 5'-TAC GTG TCG TGG AGT C-3'

Table 2. Primer sequence of RT-qPCR

Genes	Primer sequence
TNF- α	F: 5'-TCA AAC CCT GGT ATG AGC CC-3' R: 5'-ACA CCC ATT CCC TTC ACA GA-3'
MCP-1	F: 5'-CTC AGC CAG ATG CAG TTA ATG C-3' R: 5'-TCT CCA GCC GAC TCA TTG G-3'
GAPDH	F: 5'-TGC ACC ACC AAC TGC TTA G-3' R: 5'-GAT GCA GGG ATG ATG TTC-3'

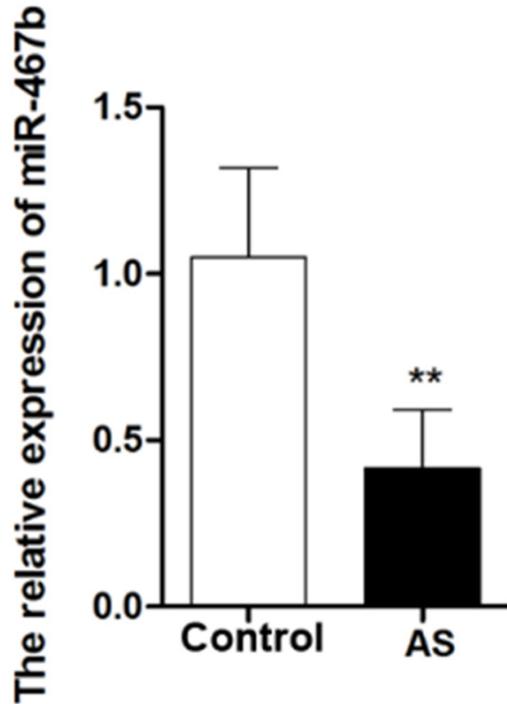


Figure 1. The expression of miR-467b in AS. Note: The expression of miR-467b in thoracic aortic tissue of mice in both AS group and control group through RT-qPCR test (**P < 0.01).

were found, such as miR-33, miR-155, miR-126-5p [8, 9]. At present, MiR-467b is a new miRNA found in the mice [10]. In this study, we detected the expression of miR-467b in aortic tissue of AS model mice and also investigated the effect of over or down-regulate expression of miR-467b on the expression of TNF- α and MCP-1.

Material and methods

Animal model of AS

20 ApoE^{-/-} mice with C57BL/6 (6-8 weeks old and weight of 20-25 g) were obtained from Peking University, Beijing, China and fed in SPF animal rooms. 20 cases of ApoE^{-/-} mice were randomly divided into the control group (n = 10) and AS model group (n = 10). The control group received a standard diet, while the mice in AS model group were given a high-fat diet (containing 0.25% cholesterol and 15% cocoa butter) for 12 weeks to induce atherosclerotic plaque. Then mice in both groups were killed and thoracic aortic tissues were collected. Atherosclerotic plaque was detected. Thoracic aortic tissue was put into liquid nitrogen for the collecting of RNA. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). Eighth Edition, 2010. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Henan Provincial People's Hospital.

Analysis of miR-467b expression in thoracic aortic tissue

Thoracic aortic tissue sample was collected, Trizol (Invitrogen, Carlsbad, CA, USA) was used, chloroform extracting method was applied to obtain total RNA, 4 μ L primed miRNA RT (500 nmol/L) was used for the template-primer mixed liquor. Such mixed liquor was treated by water bath with 70°C for 10 min, then ice bath

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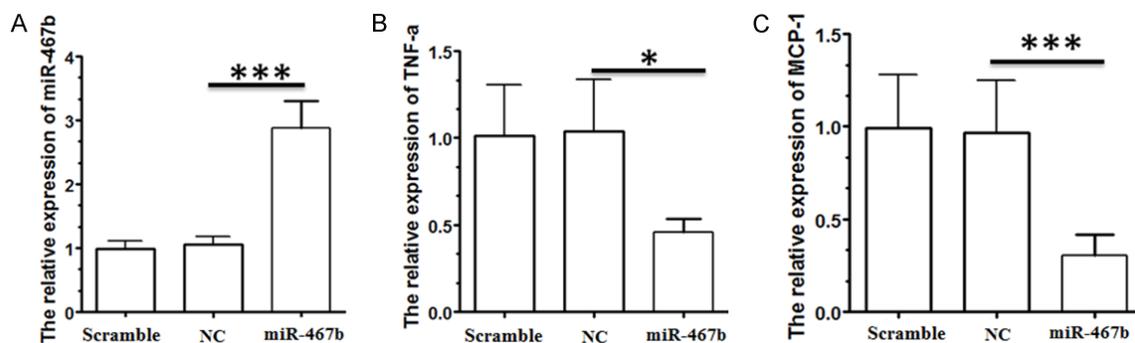


Figure 2. Over-expression of miR-467b inhibits the expression of TNF- α and MCP-1 in macrophages. A. RT-qPCR detection of miR-467b level in RAW264.7 macrophages transfected with miR-467b and control NC (* $P < 0.05$; *** $P < 0.001$); B. RT-qPCR detection of TNF- α mRNA expression in RAW264.7 macrophages transfected with miR-467b and control NC (* $P < 0.05$; *** $P < 0.001$); C. RT-qPCR detection of MCP-1 mRNA expression in RAW264.7 macrophages transfected with miR-467b and control NC (* $P < 0.05$; *** $P < 0.001$).

with 0°C for 2-3 min. The reaction system was as follows: 4 μ L dNTP (2.5 mmol/L), 10 μ L 5 \times RT Buffer, 1 μ L reverse transcriptase (200 U/mL), 1 μ L RNase inhibitor (40 U/mL). After ice bath, the template-primer mixed liquor was put into the reaction system, no-nucleic acid enzyme was used to complete the liquor to 50 μ L. Then the mixed liquor was treated by water bath with 40°C for 90 min, reverse transcription was terminated after water bath with 95°C for 5 min. Then ice bath for 5 min to obtain cDNA. This cDNA can apply to the microRNA Real-time PCR test.

Pre-denatured at 95°C for 20 s, then obtained Ct value through circulating steps of denaturation at 95°C for 10 s, anneal at 60°C for 20 s and extension at 70°C for 10 s for 40 cycles. Calculate Ct values according to the method provided by ABI PRISM 7700 Sequence Detection System User Bulletin #2 of Applied Biosystem company and compare the expression of miRNA in every sample. MiR-467 as well as the reverse transcription and quantitative primer of internal reference U6 were bought from RiboBio Co., Guangzhou, China. Primer sequence was showed in **Table 1**.

Real-time quantitative PCR (RT-qPCR)

LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfected miR-467b, NC and miR-467b inhibitor into RAW 264.7 macrophages according to the kit instructions. RAW 364.7 macrophages was bought from the cell bank of the Chinese Academy of Science (Shanghai, China). RAW 264.7 macrophages were maintained in RPMI-1640 supplement with 10% fetal bovine serum (FBS, Gibco, Grand

Island, NY, USA), 100 U/mL gentamicin, 100 μ g/mL streptomycin and 100 U/mL penicillin. All these cells were incubated in a incubator at 37°C and 5% CO₂. After 24 h, these cells were collected, RNA was extracted, cDNA was obtained through reverse transcription. RT-qPCR was preformed to test the expression of TNF- α and MCP-1. The quantitative primers were showed in **Table 2**. The primer used in experiment was synthesized by Shenergy New Energy Investment Co., Ltd., Shanghai, China.

Statistical analysis

All statistical analyses were performed using SPSS version 17.0 software (SPSS Inc, Chicago, IL, USA). The measurement data was shown as the mean \pm SD and tested by normality test. The comparison of groups was showed as the t-test. $P < 0.05$ was considered to be statistically significant.

Results

The expression of miR-467b in AS

As shown in **Figure 1**, the results showed that the presentation of thoracic aorta atherosclerotic plaques, and the expression of miR-467b in ApoE^{-/-} mice with high-fat diet was significantly down-regulated compared to the control group ($P < 0.05$) (**Figure 1**).

Over-expression of miR-467b inhibited the expression of TNF- α and MCP-1 in RAW 264.7 macrophages

As shown in **Figure 2**, the expression of TNF- α and MCP-1 in cells transfected by miR-467b

Expression of miR-467b in atherosclerosis

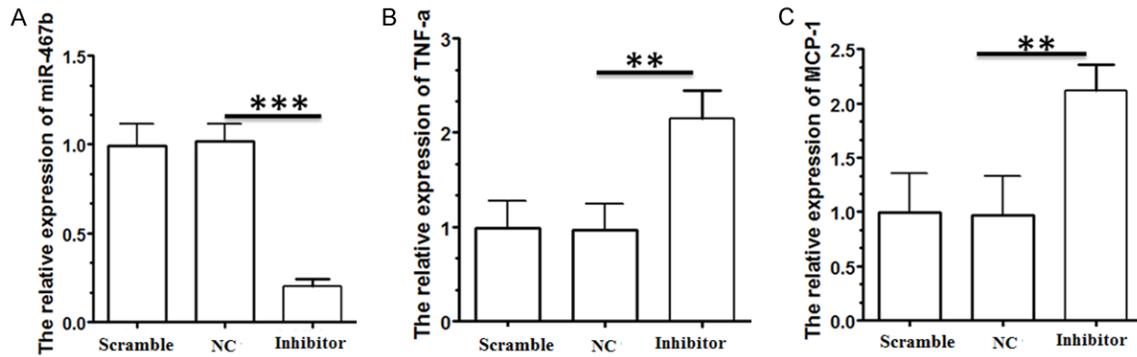


Figure 3. The inhibition of miR-467b promotes the expression of TNF- α and MCP-1 in macrophages. A. RT-qPCR detection of miR-467b level in RAW264.7 macrophages transfected with miR-467b inhibitor and control NC (**P < 0.01); B. RT-qPCR detection of TNF- α mRNA expression in RAW264.7 macrophages transfected with miR-467b inhibitor and control NC (**P < 0.01). C. RT-qPCR detection of MCP-1 mRNA expression in RAW264.7 macrophages transfected with miR-467b inhibitor and control NC (**P < 0.01).

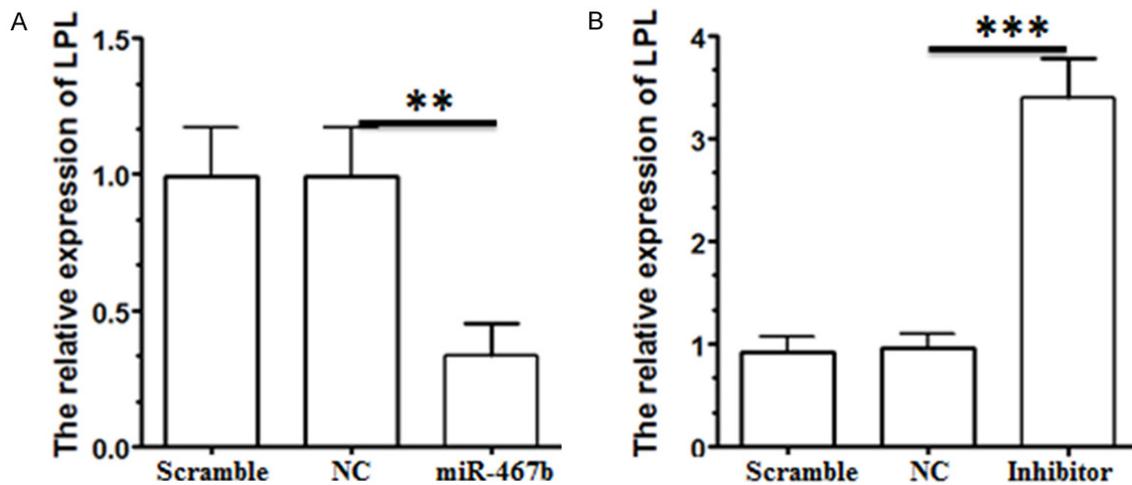


Figure 4. Prediction of target site for miR-467b. A. RT-PCR detection of LPL mRNA expression in RAW264.7 macrophages transfected with miR-467b and control NC (**P < 0.05; ***P < 0.01); B. RT-PCR detection of LPL mRNA expression in RAW264.7 macrophages transfected with miR-467b inhibitor and control NC (**P < 0.01; ***P < 0.01).

and that transfected by NC was detected by RT-qPCR. The result showed that over-expression of miR-467b obviously inhibited the expression of TNF- α and MCP-1 in RAW 264.7 macrophages. The difference was statistically significant (P < 0.05) (**Figure 2**).

The inhibition of miR-467b expression promoted expression of TNF- α and MCP-1 in RAW 264.7 macrophages

As shown in **Figure 3**, the expression of TNF- α and MCP-1 in cells transfected by miR-467b inhibitor and that transfected by NC was detected by RT-qPCR. The result shows that the inhibition of miR-467b expression promoted expres-

sion of TNF- α and MCP-1 in RAW 264.7 macrophages. The difference was statistically significant (P < 0.05) (**Figure 3**).

Predicting the target site for miR-467b

According to the above results, we found that it was not the combination of miR-467b with TNF- α and MCP-1 that directly inhibited the expression of them. Thus we predicted the target site for miR-467b by using bioinformatics software so that we can study the regulation mechanism of miR-467b for TNF- α and MCP-1. According to the result of RNAhybrid software, we predicted that miR-467 may play a role on LPL. Through detecting the expression of LPL

mRNA, we also found that over-expression of miR-467b can inhibit the expression of LPL mRNA when there was over-expression or inhibition of miR-467b in RAW 264.7 macrophages. However, when there was an inhibition of miR-467b, the expression of LPL mRNA was increased. This result prompted that LPL may be the target site for miR-467b (Figure 4).

Discussion

Atherosclerosis, a common kind of angiocardopathy, has complex pathologic process, including the induction on the expression of many inflammatory factors through the gathering of lipid. The macrophage is the main cell to promote such vascular inflammation [11, 12]. At the early stage of AS, with the function of inflammatory factors and oxidative stress, mononuclear cells will differentiate to macrophages, then they will become the lipid layer. This process above plays a critical role during the forming of atherosclerotic plaque [13, 14]. Apolipoprotein E (ApoE) induces the forming of AS through participating in the transfer and metabolism of lipid. In this investigation, we induced AS model mice by feeding ApoE^{-/-} mice with high-fat diet [15-17].

In this study, we found that the expression of miR-467b was down-regulated in the thoracic artery tissue of AS mice, which referred that the inhibition of miR-467b may help the growth of AS. Then according to the over-expression of miR-467b, we found that miR-467b can depress the expression of TNF- α and MCP-1, and the low level of miR-467b can induce the expression of TNF- α and MCP-1 during the process of AS. The analysis of miR-467b for TNF- α and MCP-1 showed that it was not the combination of miR-467b with TNF- α and MCP-1 that directly inhibited the expression of them. The prediction of bioinformatics software showed that LPL may be the target site for miR-467b. There is a high level expression of LPL in AS generally, which promoted the forming of foam cells, the expression of inflammation factors, the gathering of lipid [18, 19]. Some studies found that after the gene knock-out of LPL, atherosclerosis of mice with AS will be relieved obviously [20]. Therefore, LPL was regarded as an important factor in the process of AS. LPL in the macrophage also was regarded as a medium between glycoprotein and lipoprotein receptor, which helped the forming of thero-

sclerotic plaque through the retention and gathering of lipoprotein. In addition, LPL in macrophages stimulated the secretion of many kind of inflammatory factors. Therefore, we predicted that the down-regulated expression of miR-467b was propitious for the generation of LPL, which promoted the expression of TNF- α and MCP-1. This investigation prompts that over-expression of miR-467b may be a new approach for treating AS mice or even patients.

In conclusion, this investigation shows that there is a down-regulated expression of miR-467b in the AS mice, which may be good for the expression of LPL and promotes the participation of TNF- α and MCP-1 during the process of AS. Although it is necessary for the further study to confirm whether LPL is the target site for miR-467b, the non coding RNA in this investigation may provide a new method for the treatment of AS.

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Disclosure of conflict of interest

None.

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