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MiR-181b regulates atherosclerotic inflammation and vascular endothelial function through Notch1 signaling pathway

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Abstract. – OBJECTIVE: **To explore the influences of micro ribonucleic acid (miR)-181b on the inflammation and vascular endothelial function in atherosclerosis (AS), and its specific molecular regulatory mechanism.**

MATERIALS AND METHODS: **44 apolipoprotein E (ApoE)$^{-/-}$ 7 weeks old male rats were randomly divided into normal diet group (NC group) and AS model group (high-fat diet feeding). Rat aorta was dissected and the serum sample was collected in both groups. The serum levels of inflammatory factors in both groups were detected *via* enzyme-linked immunosorbent assay (ELISA). The mRNA levels of miR-18b and Notch1 were detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Moreover, aortic endothelial cells were extracted from AS rats. The miR-18b binding target gene was analyzed *via* bioinformatics and further verified by the Luciferase reporter gene assay. The protein expressions of miR-18b and Notch1 in endothelial cells transfected with miR-181b mimic or inhibitor were detected. Influence of miR-181b on vascular endothelial indexes was also detected.**

RESULTS: **Compared with those in the NC group, the serum levels of interleukin-6 (IL-6), IL-10, IL-13 and tumor necrosis factor-α (TNF-α) in the AS group significantly increased (p<0.05). The mRNA level of miR-18b in AS plaques was significantly lower than that in NC arterial tissues. Conversely, Notch1 level in AS plaques was markedly higher than that in the NC arterial tissues (p<0.05), with the mean difference of 2.12 and 2.82 folds (p<0.05). According to the Pearson correlation analysis, there was a significant negative correlation between mRNA levels of miR-181b and Notch1 in AS tissues (r=-0.65, p=0.014). The bioinformatics analysis showed that there were complementary binding sites between miR-181b and Notch1. The Luciferase reporter gene assay confirmed the presence of direct binding sites between miR-181b and Notch1. Western blotting revealed that the overexpression of miR-181b downregulated Notch1 and hs-CRP, but upregulated BNP (p<0.05). Opposite trends were obtained by miR-181b knockdown (p<0.05).**

CONCLUSIONS: **The decline in the miR-181b expression may be an important factor in AS plaque formation and vascular endothelial injury by regulating Notch1.**

Key Words
MiR-181b, Notch1, Atherosclerosis, Vascular endothelial function.

Introduction

In atherosclerosis (AS), the abnormal lipids are under the vascular wall intima, which mediates the formation of foam cells and further lead to vascular endothelial dysfunction and inflammatory response[1,2]. In this process, immune cells, lymphocytes and dendritic cells are activated, and some cytokines are released to further amplify the inflammatory response. Subsequently, macrophages, platelets and smooth muscle cells are activated, thereby dramatically increasing the inflammatory substances[3,4] and aggravating the plaque instability and rupture. AS greatly threatens people's quality of life and health.

The gene regulatory factor micro ribonucleic acid (miRNA) is involved in a wide range of processes closely related to AS and cardiovascular disease[5,6]. The activation of endothelial cells is a major event in the pathogenesis of AS. Recent studies[7] have clarified that the miR-186 family may be involved in the regulation of AS. MiRNAs in AS regulation can serve as targets for the development of novel anti-AS therapeutic strategies. It has been proved in this paper that miR-181b, as a protective factor in AS, was significantly down-regulated in AS plaques, which resisted AS by negatively regulating Notch1. The present study aims to provide new clues for finding the role of miRNAs in AS plaque formation and cardiovascular therapy.

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Materials and Methods

Animal Model

44 apolipoprotein E (ApoE)$^{-/-}$ 7 weeks old male rats were fed in the specific pathogen-free (SPF) environment and had free access to food and water. The rats were randomly divided into normal diet group (NC group, n=22) and AS model group (high-fat diet feeding, n=22). After 2 months, rats were decapitated and the samples were collected. The morphology of aortic tissues in the NC group and AS model group was detected *via* hematoxylin-eosin (HE) staining (Boster, Wuhan, China). All animal investigations strictly conformed to the guidelines for the use and care of laboratory animals of the National Animal Ethics Institute. This study was approved by the Animal Ethics Committee of the Liaocheng People's Hospital Animal Center.

Detection of Inflammatory Factors and Endothelial Function Indexes Via Enzyme-Linked Immunosorbent Assay (ELISA)

The serum levels of interleukin-6 (IL-6), IL-10, IL-13, tumor necrosis factor-α (TNF-α), endothelin-1 (ET-1), brain natriuretic peptide (BNP) and high-sensitivity C-reactive protein (hs-CRP) were detected *via* horseradish peroxidase-labeled immune antibody sandwich assay. Briefly, the antibodies against IL-6 (RD, article No.: D6050, New York, NY, USA), IL-10 (RD, article No.: DY417), IL-13 (RD, article No.: M1300CB), TNF-α (RD, article No.: 210-TA), ET-1 (RD, article No.: QET00B), BNP (RD, article No.: MAB36041) and hs-CRP (RD, article No.: DCRP00) were coated into each well of a 96-well plate. Cells were incubated with antibodies in an appropriate amount of serum. The content of enzyme-binding TNF-α was detected with tetramethylbenzidine as a substrate. The optical density (OD) value was read at the dual wavelengths of 450 nm and 600 nm using a microplate reader, and the concentration of samples was calculated.

Detection of mRNA Levels of MiR-181b and Notch1 Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was extracted from rat aorta using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA concentration was immediately measured using a spectrophotometer. According to the experimental procedures (TaKaRa, Otsu, Shiga, Japan), 1 μg RNA was loaded for reverse transcription. The complementary deoxyribonucleic acid (cDNA) obtained was stored at -20°C, and the mRNA level of each index was detected according to the instructions of the All-in-One™ qPCR Mix kit (GeneCopoeia, Inc., Rockville, MD, USA). The primer sequences were as follows: MiR-181b: sense: 5'-TGCGCTAGCACCATCTGAAAT-3', antisense: 5'-CAGTGCAGGGTCCGAGGT-3'. Notch1: sense: 5'-TCCTCAGGTTGGCACAGGTG-3', antisense: 5'-CACGTGTCGGTCAGTCCTCA-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The reaction conditions were as follows: pre-denaturation at 95°C for 15 min, 94°C for 20 s and 60°C for 34 s, a total of 40 cycles. The melting curve was analyzed. Three repeated wells were set for each sample, and the relative expression level was expressed by $2^{-\Delta\Delta Ct}$.

Extraction of Primary Aortic Cells

The aorta was digested with Dispase II (Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight using LS column and magnetic frame (Miltenyi, Bergisch Gladbach, Germany) according to standard protocols. Dermis tissues were collected, placed into a 15 mL centrifuge tube and added with 5 mL of pre-heated collagenase (Sigma-Aldrich, St. Louis, MO, USA), followed by digestion for 1 h. After the tissues were filtered using a 200-mesh gauze, the medium was added for centrifugation for 5 min. Finally, the precipitated cells were target cells.

Luciferase Reporter Gene Assay

The pmir-vector (containing vector plasmid), pmir-Notch1-WT (containing a miR-181b binding site predicted) and pmir-Notch1-Mut (containing a mutant miR-181b 5'UTR binding site) were constructed by Shanghai KeyGEN Biotech Co., Ltd. (Shanghai, China). Luciferase activity in lysis buffer of HEK293-T cells co-transfected with the above plasmids was determined using the Luciferase reporter gene assay kit (Applied Biosystems, Foster City, CA, USA). Luciferase activity of miRNA negative control (control) or miRNA-181b at 48 h was determined using the Dual-Luciferase reporter gene analysis system (Promega, Madison, WI, USA).

Detection of Notch1 Expression Via Western Blottin

Transfected cells were lysed in 1×radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The protein samples were separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF)

Table I. Expression of serum inflammatory factors in both groups.

Group	NC group (n=22)	AS group (n=22)	*t*	*p*
IL-16 (pg/mL)	12.15±7.43	45.22±15.42	8.51	<0.05
IL-10 (pg/mL)	33.74±6.32	43.07±12.32	5.27	<0.05
IL-13 (pg/mL)	26.24±8.45	46.36±13.57	4.45	<0.05
TNF-α (pg/mL)	35.07±10.03	77.19±14.26	11.23	<0.05

membrane (Millipore, Billerica, MA, USA) and incubated with the primary antibody against Notch1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The relative protein expressions were detected *via* the OD analysis, with GAPDH as the internal reference (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical Analysis

GraphPad Prism software (Version 5.01, GraphPad Software, La Jolla, CA, USA) was used for analysis. Measurement data were expressed as ($\bar{\chi}$±s), and Student's *t*-test was used to analyze the difference between the observation group and control group. The correlation between miR-181b and Notch1 expression in AS plaques was detected *via* Pearson correlation analysis. $p<0.05$ suggested that the difference was statistically significant.

Results

Pathological Morphology of AS Rats Detected Via HE Staining

Rat aorta tissues in both groups were prepared into frozen sections, and the morphology was detected *via* HE staining. It can be seen under a microscope that the aorta in the NC group had normal morphology without inflammatory cell aggregation. In the AS model group, there were foam cells and significant lipid deposition under the intima, and inflammatory cell infiltration in some inner vascular walls, indicating the successful establishment of the AS model.

Expression of Serum Inflammatory Factors in Both Groups Detected Via ELISA

The serum levels of inflammatory factors in both groups were detected *via* ELISA. As shown in Table I, the levels of IL-6, IL-10, IL-13, and TNF-α in the AS group were significantly higher than those in the NC group ($p<0.05$).

MiRNA-18b and Notch1 Expression Detected Via RT-PCR

The mRNA levels of miR-18b and Notch1 in aortic tissues in both groups were quantitatively detected *via* RT-PCR. As shown in Figure 2, the mRNA level of miR-18b in AS plaques was significantly lower than that in NC arterial tissues. However, Notch1 level in AS plaques was significantly higher than that in NC arterial tissues ($p<0.05$), with the mean difference of 2.12 and

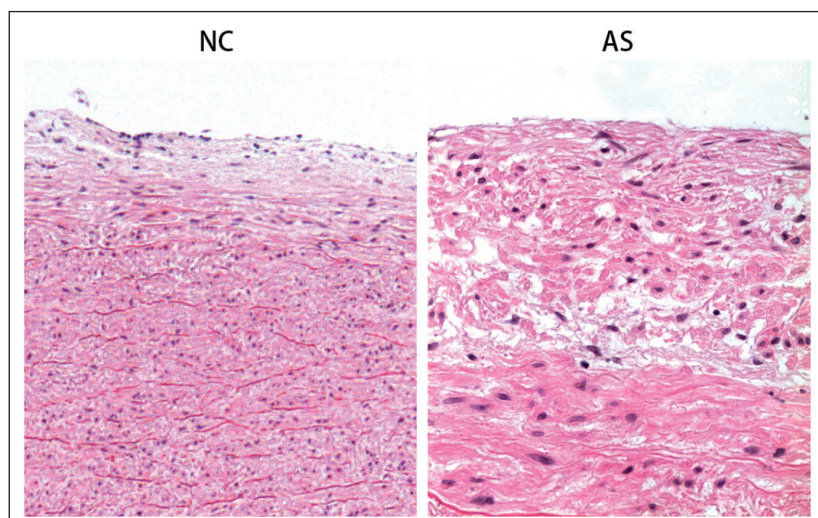

Figure 1. Pathological morphology of AS rats detected via HE staining (×200). The aorta in NC group has normal morphology. In the AS model group, there are foam cells and significant lipid deposition under the intima, and inflammatory cell infiltration in some inner vascular walls.

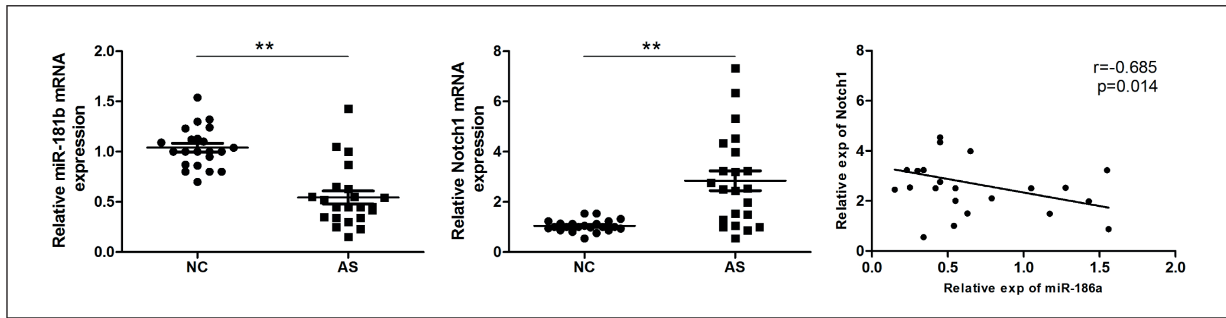

Figure 2. MiRNA-18b and Notch1 expression detected via RT-PCR.

2.82 folds (p<0.05). According to the correlation analysis, there was a significant negative correlation between mRNA levels of miR-181b and Notch1 in AS tissues (r=-0.65, p=0.014).

Binding Target Genes Analyzed Via Bioinformatics

Through bioinformatics analysis (http://targetscan.org/, http://www.mirdb.org), it was found that there were complementary binding sites between miR-181b and Notch1 3'UTR (Figure 3A). We first isolated aortic endothelial cells from AS rats, and constructed Notch1 wild-type and mutant-type plasmids. Dual-Luciferase reporter system confirmed that there were direct binding sites between Notch1 and miR-181b (Figure 3B).

Influence of MiR-181b on Notch1 Expression

To further verify the regulatory correlation of miR-181b on Notch1, the primary endothelial cells were transfected with the empty sequence,

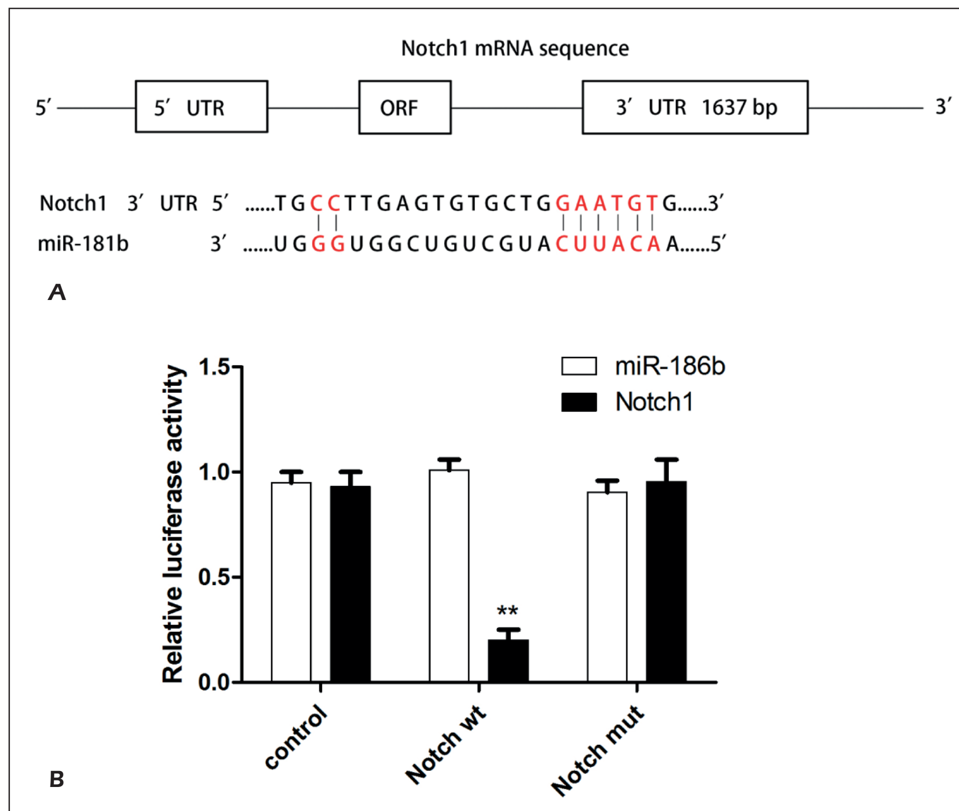

Figure 3. Binding target genes analyzed via bioinformatics. **A**, Binding sites between miR-181b and Notch1 analyzed via bioinformatics. **B**, it is confirmed in Dual-Luciferase reporter gene assay that Notch1 is a binding target gene of miR-181b.

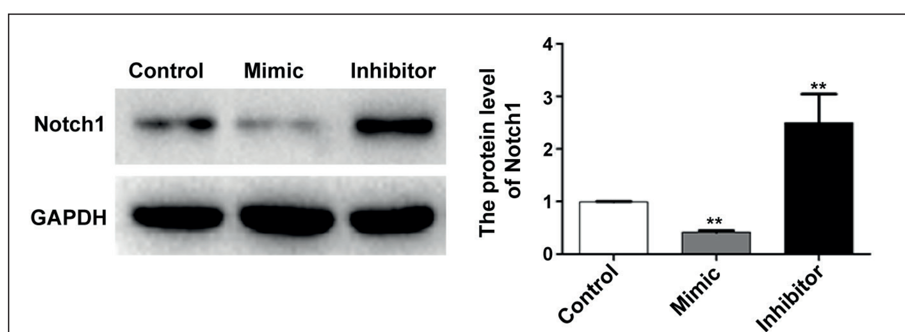

Figure 4. Influence of miR-181b on Notch1 expression detected via Western blotting. After overexpression of miR-181b, the Notch1 protein abundance markedly declines (p<0.05). The Notch1 protein in inhibitor group is remarkably increased compared with that in the NC group (p<0.05).

miR-181b mimic or inhibitor, respectively. After overexpression of miR-181b, protein abundance of Notch1 significantly declined (p<0.05). The protein level of the Notch1 in the inhibitor group was markedly increased compared with that in the NC group (p<0.05), suggesting that miR-181b had a negative regulatory correlation on Notch1 (Figure 4).

Changes in ET-1, BNP and Hs-CRP Levels

Compared with those in the NC group, the protein levels of ET-1 and BNP were remarkably increased, while the hs-CRP level markedly decreased in the mimic group (p<0.05). There were opposite trends in the inhibitor group, manifesting as downregulated protein levels of ET-1 and BNP, and upregulated hs-CRP level (p<0.05) (Table II).

Discussion

AS is a kind of chronic inflammatory state, and its progression is closely controlled by innate and adaptive immunity. The clinical acute events are usually caused by plaque rupture and thrombosis. According to the pathological studies, the development of thrombosis-mediated acute events mainly depends on the plaque composition and vulnerability[8]. Recent research has demonstrated that miRNAs play important roles in the pathogenesis of vascular and inflammatory diseases, including stroke and plaque rupture. The miR-181 family plays a key role in cardiovascular inflammation, atherosclerotic plaque formation and acute stroke events[9-11]. In rats with focal ischemic stroke, miR-181b expression declines with the shortened distance from the ischemic core[12]. The increase in miR-181b expression can lower the total arterial load. In the present work, it was found that the mRNA level of miR-181b in arterial plaques in AS model group was significantly lower than that in NC group, which was consistent with previous findings. At the same time, the serum levels of inflammatory factors in AS model group significantly increased, which were consistent with the research results of Busch et al[13] that miR-181b exerts a pro-inflammatory effect on endothelial cells. They believed that miR-181b is related to the formation of AS plaque by promoting monocyte adhesion and activating inflammatory cytokines and chemokines. It was found in previous studies[14,15] that there are large numbers of macrophages in vulnerable plaques, which are very important for plaque rupture. Moreover, miR-181b can also reduce the plaque area, necrotic area and macrophage infiltration *via* regulating the macrophage polarization. However, its specific regulatory mechanism has not been clarified yet.

Table II. Expression of endothelial function indexes in supernatant detected via ELISA.

Group	NC group	Mimic group	Inhibitor group
ET-1 (ng/L)	65.85±15.08	81.24±21.50	110.54±32.54
BNP (ng/L)	245.73±132.44	330.82±140.32	155.34±83.4
Hs-CRP (mg/L)	3.44±0. 87	1.19±0.56	6.83±2.24

The complex miRNA regulatory events are included in the known transcription factors and signal networks that control cell fate and differentiation. Multiple genes in different cell types have been determined as direct targets of miR-181b[16,17]. Several studies have demonstrated that there is a correlation between miR-181b and Notch1. Notch1 signaling suppressor is the target of miR-181b, which is related to the cellular stress response in several tumors[18,19]. The abnormal activation of Notch1 signal is correlated with the macrophage dysfunction, and Notch1 plays a key role in determining M1 and M2 polarization of macrophages[20]. In the present work, the miR-181b base sequence was compared using crossover analysis databases (miRBase and Targetscan) to determine the target gene of miR-181b. It was found that there were complementary binding sites between miR-18b and Notch1 3'UTR, indicating that Notch1 may be the target of miR-181b. Furthermore, in primary endothelial cells isolated from AS plaques, Luciferase reporter gene assay confirmed that Notch1 was a direct target of miR-181b, which were also consistent with the correlation analysis results (Figure 2C). In addition, cells were transfected with miR-181b mimic and inhibitor to evaluate its function in AS. A negative regulatory correlation between Notch1 and miR-181b was identified. In other words, the overexpression of miR-181b *in vitro* significantly inhibits the expression of Notch1. To explore the influence of the miR-181b/Notch1 signaling pathway on vascular endothelial cells, the protein levels of ET-1, BNP and hs-CRP in primary cell supernatant were detected. It was found that the overexpression of miR-181b protected the endothelial cell function by inhibiting Notch1 expression.

Conclusions

We showed that the inhibition on miR-181b, the protective factor in AS, leads to the increased expression of Notch1, and it may be correlated with the AS-activated inflammatory factors. Our results provide new clues for finding the role of miRNAs in AS plaque formation and cardiovascular therapy.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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