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MiR-126 promotes endothelial cell apoptosis by targeting PI3K/Akt in rats with lower limb arteriosclerosis obliterans

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Abstract. – OBJECTIVE: To investigate the influence of micro ribonucleic acid (miR)-126 on the rats with lower limb arteriosclerosis obliterans (ASO).

MATERIALS AND METHODS: Male Sprague-Dawley rats aged 3 months old were randomly divided into Sham operation group (Control group, n=10) and Model group (n=10), and the model of lower limb ASO was established. After modeling, the expression of miR-126 in arteries was detected using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), and the change in the downstream signaling pathway was examined *via* Western blotting. The human umbilical vein endothelial cells (HUVECs) were induced by oxidized low-density lipoprotein (Ox-LDL) to establish the model of endothelial injury, followed by detection of miR-126 expression. Then, the Luciferase assay was performed to verify the downstream target gene of miR-126. After being cultured, HUVECs were set as Control group, Ox-LDL induction group, and Ox-LDL + miR-126 inhibitor group, and the expressions of phosphorylated-protein kinase B (p-Akt) and cleaved cysteine-aspartic protease-3 (Caspase-3) were detected in the above groups.

RESULTS: After the establishment of the model, the expression level of miR-126 was raised in vessels, but the phosphatidylinositol 3-hydroxy kinase (PI3K)/Akt signals were weakened ($p<0.01$). Ox-LDL-induced endothelial cell apoptosis promoted the expression of miR-126, and the difference was statistically significant. The bioinformatics analysis results showed that PI3KR2 was a direct target of miR-126, which was also proven *via* the Luciferase assay. Moreover, the transfection with miR-126 inhibitor into endothelial cells suppressed Ox-LDL-induced cell apoptosis, thereby persistently activating the PI3K/Akt signaling pathway ($p<0.01$).

CONCLUSIONS: In rats with lower limb arteriosclerosis obliterans (ASO), miR-126 represses the PI3K/Akt signaling pathway to accelerate endothelial cell apoptosis.

Key Words:
MiR-126, P-Akt, Lower limb arteriosclerosis obliterans.

Introduction

As a leading cause of limb loss in adults around the world, lower limb arteriosclerosis obliterans (ASO) is characterized by the accumulation of fibrous elements and lipids in the large arteries, resulting in various life-threatening complications[1]. Once vessels are destroyed externally, the vascular endothelial injury will occur and then induce the proliferation and migration of smooth muscle cells, thereby causing luminal stenosis and impairing vascular function[2,3]. However, the specific molecular mechanism in ASO remains unknown now.

The endothelial cells, the foundation of vessels, can maintain both the integrity of vessels and the stability of the whole vascular system[4]. Endothelial dysfunction, which is largely attributed to apoptosis, is regarded as one of the initial pathological steps and plays a major role in the progression of ASO. It is generally suggested that the status of the endothelial cells is affected by numerous cardiovascular risk factors and different stimuli, among which the low-density lipoprotein (LDL) is considered as a risk factor[5]. After oxidation, LDL is transformed into oxidized LDL (Ox-LDL) to induce endothelial injury and further spur the initiation and progression of ASO[6].

Phosphatidylinositol 3-hydroxy kinases (PI3Ks), the upstream signaling molecules of protein kinase B (PKB/Akt), activate Akt to regulate the changes in the intracellular signaling pathways and cell functions[7]. Tsoyi et al[8] have revealed that the activation of the PI3K/Akt signaling pathway can

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modulate the migration, proliferation, and apoptosis of the endothelial cells. Besides, the PI3K/Akt signals are markedly reduced in Ox-LDL-induced endothelial cells, but the specific regulatory mechanism has not been clarified yet. The high expression of micro ribonucleic acid (miR)-126, a miRNA specifically expressed in endothelium, can suppress vascular inflammation, whereas its low expression can destroy the integrity of vessels[9]. In addition, it has been predicted that PI3K contains the miR-126 binding site.

Based on the research above, it is speculated in this study that miR-126 probably regulates the PI3K/Akt signaling pathway to modulate vascular endothelial function, thereby participating in lower limb AOS. Therefore, the present study preliminarily explored the relationship between them in the regulation of ASO in rats.

Materials and Methods

Establishment of Rat Model

A total of 20 male Sprague-Dawley rats were purchased from Fudan University Animal Center and randomly divided into Sham operation group (Control group, n=10) and Model group (n=10). This study was approved by the Animal Ethics Committee of Fudan University Animal Center. Each of the rats was fed with a high-fat diet in separate cages for 1 week, and then they were anesthetized and disinfected on the left hind limb. Subsequently, the skin was cut open from the groin until below the knee to pinpoint the bifurcation of the femoral artery at the popliteal artery. With the distal femoral artery blocked using an artery clamp, 0.2-0.3 mL of ddH_2O was syringed into the blocked artery until vascular filling. After injection for 5 min the rats were revived, followed by hemostasis and suture.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in a vascular cell basal medium containing 10% fetal bovine serum (FBS) and 1% double antibodies (Gibco, Rockville, MD, USA) at 37°C and with 5% CO_2.

Western Blotting

After the cells were treated, the proteins were sampled using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) on ice and quantified. Then, the proteins were diluted with buffer solution, loaded for electrophoresis and transferred onto a membrane. Subsequently, the resulting proteins were sealed in 5% skim milk for 2 h and incubated with the primary antibody (Abcam, Cambridge, MA, USA) at 4°C overnight. After the primary antibody was recycled, the membrane was washed and added with the secondary antibody (Abcam, Cambridge, MA, USA) for incubation at room temperature for 1 h. Finally, the chemiluminescence color development was conducted.

Cell Transfection and Treatment

Firstly, the cells were cultured in a 6-well plate (Corning, Corning, NY, USA) and grew until about 80% confluency. Then, the medium was replaced with a serum-free medium, and the cells cultured were transfected with miR-126 inhibitor or inhibitor control (Thermo Fisher Scientific, Waltham, MA, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection, the medium was added with 100 μg/mL Ox-LDL for incubation to stimulate the cells.

Luciferase Assay

The binding site of miR-126 was predicted using TargetScan and miRanda. To establish a Wild-Type (WT) Luciferase reporter plasmid PI3K regulatory subunit 2 (PI3KR2; Ruibao Biotechnology, Co., Ltd., Guangzhou, China), the binding segments were chemically synthesized, amplified, and cloned into Firefly Luciferase reporter plasmids. The binding site was then transformed into a MUT Luciferase reporter plasmid PI3KR2. In the Luciferase assay, 293T cells were seeded into a 24-well plate at a certain density and co-transfected with miR-126 or NC and WT or MUT plasmid PI3KR2. After 48 h of culture, the activity of Luciferase was determined using Dual-Luciferase Reporter Assay (E2920, Promega, Madison, WI, USA).

Determination of Cell Viability Via Cell Counting Kit-8 (CCK-8)

HUVECs were inoculated into a 6-well plate at 2.5×10^4 cells/well for transfection, and 24 h later, the cells were digested and plated in a 96-well plate at 3×10^3 cells/well. The viability of the cells was measured *via* CCK-8 (Dojindo, Kumamoto, Japan). After culture for 24, 48, or 72 h in a UV-6100 96-well plate (Shanghai Mapada Instruments Co., Ltd., Shanghai, China), the absorbance at the wavelength of 450 nm represented the relative number of viable cells. The experiment was repeated 3 times.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

As specified by the manufacturer, the total RNAs were extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA (1 μg) in each sample was reversely transcribed into complementary deoxyribonucleic acid (cDNA) using TruScript first-strand cDNA synthesis kit and gDNA eraser, and then amplified using 2×SYBR Green Master Mix according to the manufacturers' specifications (Kangwei Biotech Co., Ltd., Qitaihe, China).

Statistical Analysis

All the data were expressed as ($\bar{x} \pm s$). The Statistical Product and Service Solutions (SPSS) 12.0 software (SPSS, Chicago, IL, USA) was used in this study. The independent-samples *t*-test was adopted for intergroup comparisons. $p<0.005$ was considered statistically significant.

Results

Modeling of Lower Limb ASO in Rats

After modeling, 2 out of 20 rats died. The vascular sections of the rats in both Model group and Control group were taken, and it was discovered that the rats in the Control group had normal vessels, while those in the Model group completely obstructed vessels, indicating that the ASO model was successfully established (Figure 1).

Changes in Akt Signals and MiR-126 After Modeling

The total proteins were extracted from the Control group and Model group and subjected to electrophoresis, and the results revealed that the Model group had a significantly lower expression level of phosphorylated-Akt (p-Akt) than the Control group ($p<0.01$) (Figure 2A). Additionally, the total RNAs were extracted from the vessels and subjected to fluorescence qRT-PCR. The results revealed that the expression level of miR-126 in the Model group was substantially higher than that in the Control group ($p<0.01$) (Figure 2B).

Ox-LDL Inhibited the Viability of HUVECs

Previous studies have manifested that Ox-LDL can induce endothelial cell injury to simulate the occurrence of ASO, while some investigations have suggested that the induction by Ox-LDL will weaken the viability of HUVECs, thus leading to apoptosis. In the present study, it was corroborated through CCK-8 assay that Ox-LDL can induce a decrease in the viability of HUVECs, showing a statistically significant difference ($p<0.01$) (Figure 3).

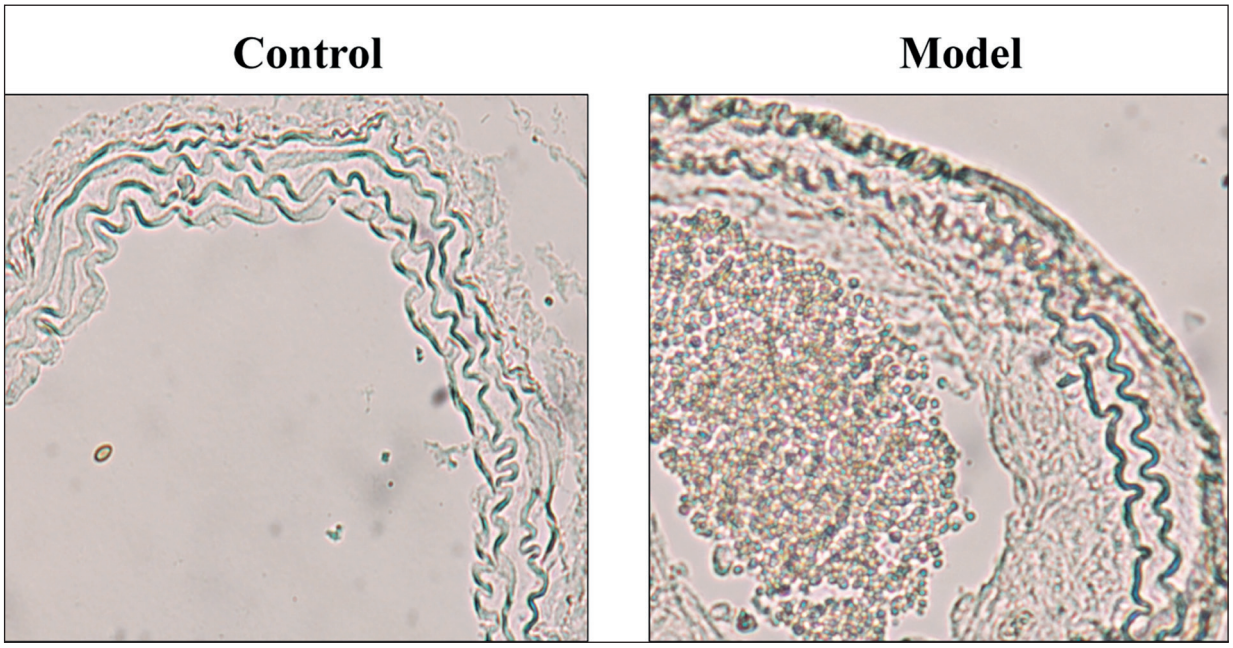

Figure 1. Morphologic changes in tissues according to the vascular sections taken after the establishment of lower limb ASO model in rats (magnification ´ 10).

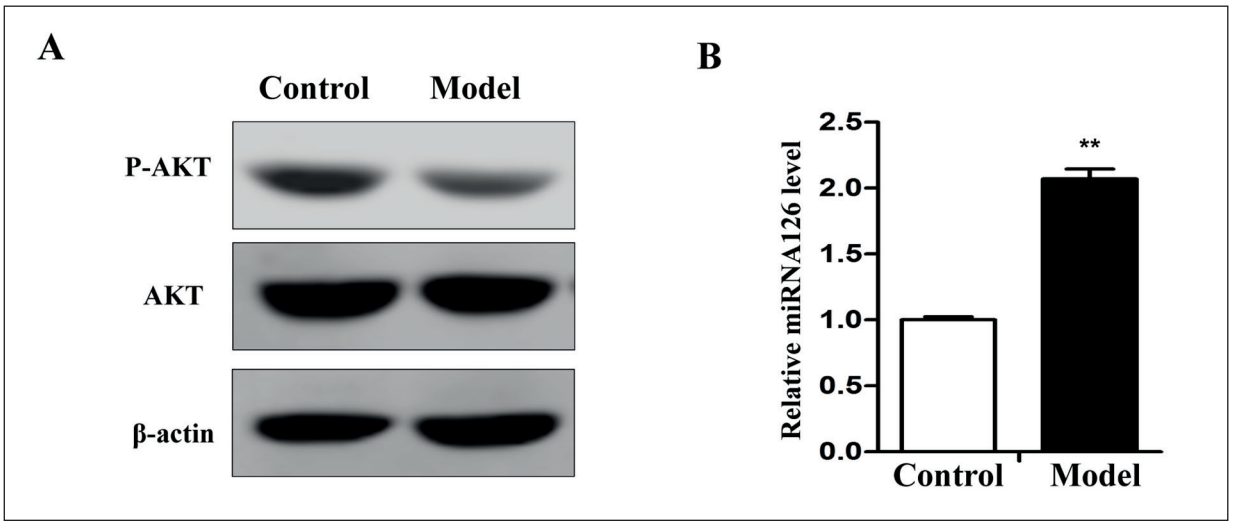

Figure 2. ***A,*** Changes in Akt signals in both Model group and Control group detected *via* Western blotting. ***B,*** Expression level of miR-126 determined *via* RT-PCR after modeling ($^{**}p<0.01$).

Ox-LDL Induced Cell Apoptosis to Promote the Expression of MiR-126

When cell apoptosis occurs, the expressions of intracellular apoptosis-related proteins are elevated, and the high expression of cleaved Caspase-3 is a major indicator for increased apoptosis. The measurement results of the protein level manifested that, compared with those in the Control group, the apoptotic cells were substantially increased in the Ox-LDL induction group after treatment with Ox-LDL (Figure 4A). Meanwhile, the RT-PCR results of the total RNAs extracted revealed that the Ox-LDL-induced endothelial injury also remarkably raised the expression level of miR-126 ($p<0.01$) (Figure 4B).

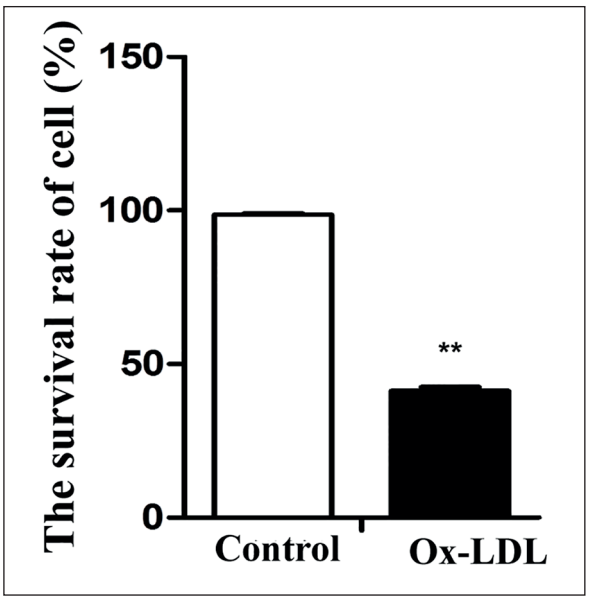

Figure 3. Viability of HUVECs detected *via* CCK-8 after treatment with Ox-LDL ($^{**}p<0.01$).

MiR-126 Target Gene Analysis

It was discovered through the bioinformatics analysis that the 3' UTR of PI3KR2 gene was complementary to miR-126 (Figure 5) and predicted to be the direct target of miR-126.

Inhibition on MiR-126 Expression in HUVECs and Verification of its Target Gene

In this study, the expression of miR-126 was first suppressed using its inhibitor in HUVECs. Then, the level of RNAs in the cells was determined, and the results indicated the successful inhibition (Figure 6A) ($p<0.01$). After the WT and MUT PI3KR2 plasmids were constructed, the cells were transfected with miR-126 inhibitor and WT PI3KR2 plasmid, and it was found that the downstream signal of Luciferases was enhanced, while it was weakened after the co-transfection with miR-126 inhibitor and MUT PI3KR2 plasmid (Figure 6B).

Relationships of MiR-126 With the Expressions of p-Akt and Cleaved Caspase-3

HUVECs with Ox-LDL-induced damage exhibited a decreased expression level of p-Akt,

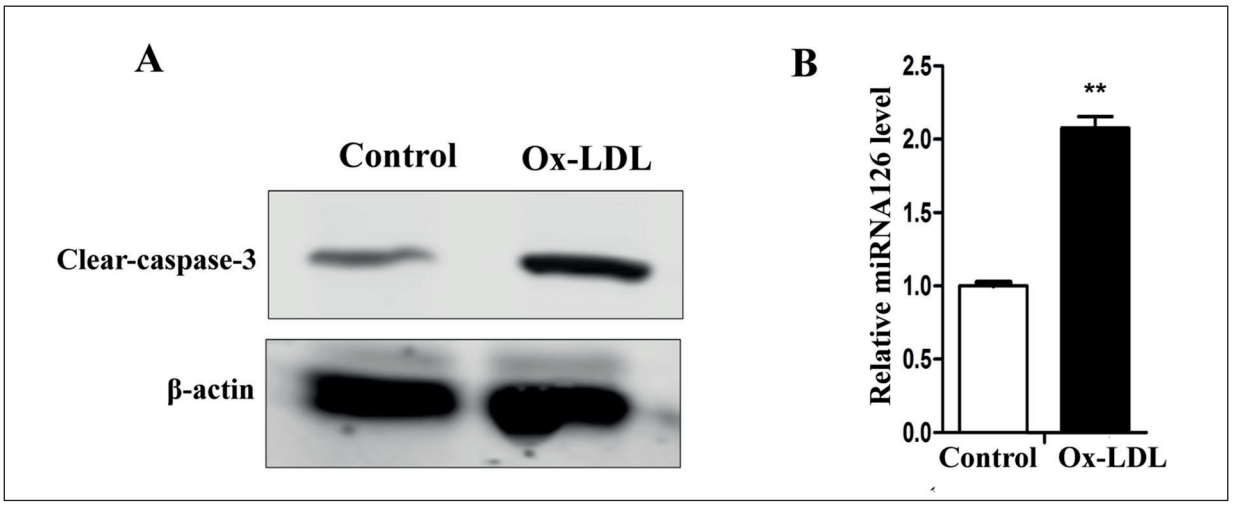

Figure 4. ***A,*** Expression of the apoptosis-related protein cleaved Caspase-3 detected *via* Western blotting. ***B,*** Expression of miR-126 in Ox-LDL-treated cells detected *via* qRT-PCR ($^{**}p<0.01$).

but a raised expression level of the apoptosis-related protein cleaved Caspase-3. In this study, the miR-126 inhibitor was used to repress the expression of miR-126 in HUVECs, and then the changes in the relevant signaling pathway were observed. It was discovered that the inhibition on miR-126 expression reversed the Ox-LDL-induced decrease in the expression level of p-Akt and reduced the Ox-LDL-induced expression of cleaved Caspase-3, thus inhibiting cell apoptosis ($p<0.01$) (Figure 7).

Discussion

It is reported that miR-126 is a small specific RNA highly expressed in the endothelial cells and affects the physiological and pathological processes of the vascular diseases as well[10]. ASO is a complex chronic inflammation and metabolic disease, and its onset is closely related to the pathological progression in vessels[11]. A leading cause of ASO is the deposition of dead cells and cellular matrix due to the apoptosis of endothelial cells externally damaged. MiR-126 has been found to be differentially expressed in the clinical cases of vascular diseases collected and can serve as an indicator for the occurrence of such diseases, suggesting that it plays an important role in vessels[12]. MiR-126 negatively regulates the expression of VCAM-1 in endothelial cells and suppresses the adhesion of macrophages to the epithelium, resulting in vascular injury[13]. In addition, analyses have suggested that miR-126 bears a close relationship with cell apoptosis, but the specific mechanism remains unclear. In this study, it was found that the expression level of miR-126 was elevated in the vessels in the rat model of the lower limb arterial injury, but that of p-Akt was lowered.

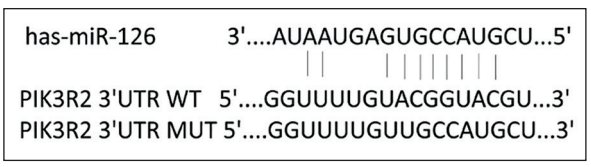

Figure 5. Bioinformatics analysis of the downstream target gene of miR-126.

In the induction of ASO *via* endothelial injury, the endothelial cells act as key players in cellular behaviors such as the recruitment of monocytes and cell migration, proliferation, and apoptosis. Endothelial injury-induced cell apoptosis is the prerequisite of ASO[14,15]. Based on previous research, the present study aims to explore the role of miR-126 in the onset of ASO by establishing the endothelial injury model *in vitro*. Ehrenreich et al[16] have demonstrated that in endothelial injury, the apoptosis of the endothelial cells is a major cause of ASO. The results of the present experiment showed that Ox-LDL induced endothelial injury to simulate the vascular injury *in vivo*. ASO has an important feature, namely the responses of cell viability and apoptosis to the stimulation by Ox-LDL or other risk factors[17]. According to the results of the present study, the stimulation by Ox-LDL prominently lowered the

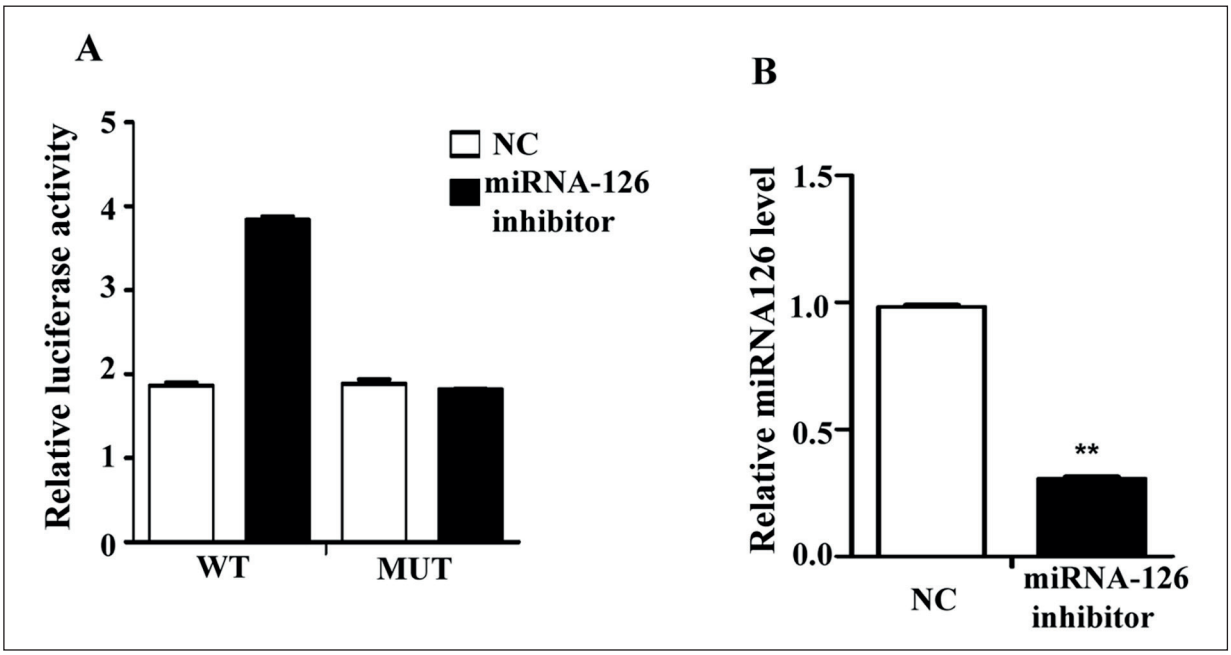

Figure 6. ***A,*** HUVECs transfected with miR-126 inhibitor, ***B,*** The upstream-downstream relationship between miR-126 and PI3KR2 verified *via* Luciferase Assay. ($^{**}p<0.01$).

viability of HUVECs and spurred the apoptosis of them, which are consistent with those of the previous analyses. Moreover, it was found in the *in vitro* experiment that the expression level of miR-126 was substantially raised in the Ox-LDL-induced HUVECs, which is consistent with the results of the *in vivo* experiment.

The PI3K/Akt signaling pathway regulates cell apoptosis in mammals, and it plays important roles in the occurrence and development of tumors, angiogenesis, and other physiological processes[18,19]. At the same time, this pathway is a crucial regulator of nuclear factor-κB (NF-κB) activation signals. Some investigations have proven that the transcription factor NF-κB is sensitive to oxidants, such as Ox-LDL. According to Feng et al[20], Ox-LDL can inhibit the PI3K/Akt signaling pathway to induce the apoptosis of human vascular endothelial cells, but the relationship between miR-126 and the PI3K/Akt signaling pathway has not yet been clarified now.

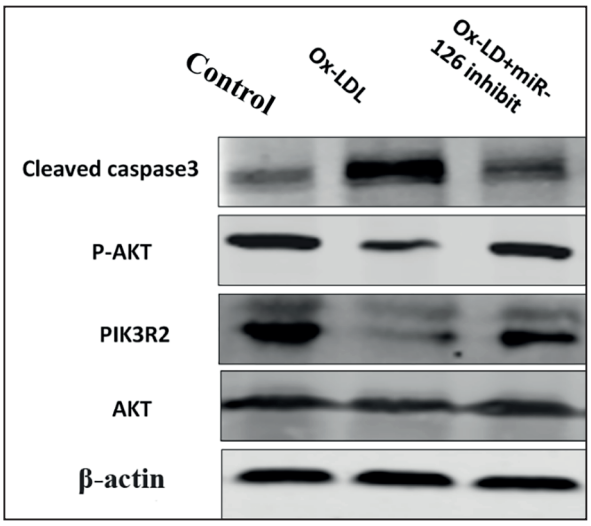

Figure 7. Expressions of p-Akt and cleaved Caspase-3 detected after inhibition on the expression of miR-126.

In the present research, the data analysis results showed that PI3K subunit p85β could be regulated in a targeted manner, and the results of the Luciferase reporter analysis confirmed the targeted reaction between miR-126 and p85β. It has been reported in the previous studies that the stimulation by Ox-LDL in HUVECs can notably inhibit the PI3K/Akt signaling pathway by a regulatory mechanism that is still unknown. According to the findings in the further experiment, the knockdown of miR-126 by adenovirus infection increased the expression level of p-Akt in Ox-LDL-treated HUVECs, thus inhibiting cell apoptosis.

Additionally, in this study, the model of the balloon injury *in vivo* confirmed that miR-126 plays an important role in lower limb ASO and

contributes to Ox-LDL-induced cell injury *via* inhibition on the activation of the PI3K/Akt signaling pathway. These findings indicate the important role of miR-126 in lower limb ASO and provide a reliable theoretical basis for its treatment.

Conclusions

This study demonstrated that, in rats with lower limb arteriosclerosis obliterans (ASO), miR-126 represses the PI3K/Akt signaling pathway to accelerate endothelial cell apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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