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Original Article

MiR-21 facilitates the proliferation of smooth muscle cells and atherosclerotic plaque formation by suppressing PTEN to activate AKT

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Abstract: Atherosclerosis (AS) is the major reason for coronary artery disease, cerebral infarction, and peripheral vasculopathy. Lipid metabolic disorder is the pathological basis for AS but but the reasons for this are unclear. MicroRNA (miR)-21 showed differential expression in AS and is related to cell proliferation. This study investigated the expression and effect of miR-21 on AS mice and on the AKT signal pathway. This study utilized LDLr-/- mice to generate an AS model, on which miR-21 expression and its effects on plaque formation were examined. Smooth muscle cells treated with oxLDL were used to investigate the targeted regulation between PTEN and miR-21, along with miR-21's effects on the AKT signal pathway and cell proliferation. The AS model mice showed significantly higher miR-21 expression ($P < 0.05$ comparing to control group), accompanied by a lower PTEN expression and an activated AKT signal pathway. The inhibition of miR-21 expression alleviated plaque injury, suggesting the involvement of miR-21 in AS plaque formation. An MTT assay and Western blot found that miR-21 suppressed PTEN expression, activated the AKT signal pathway, and inhibited smooth muscle cell proliferation. A luciferase reporter gene assay confirmed the targeted inhibition of PTEN by miR-21. In summary, MiR-21 facilitates smooth muscle cell proliferation and AS plaque formation possibly by suppressing PTEN and activating AKT. The downregulation of miR-21 might be a novel therapeutic approach for treating AS.

Keywords: Atherosclerosis, MicroRNA-21, PTEN, smooth muscle cell proliferation

Introduction

Atherosclerosis (AS) is the chief cause of coronary heart disease (CHD), cerebral infarction, and peripheral vasculopathy [1]. AS is a progressive and chronic artery disorder, manifested by a complete or partial blockade of arterial blood flow by deposited fatty acid [2]. The precise reason underlying AS, however, is still uncertain, and includes diabetes, hypertension, and hyperlipidemia as high risk factors.

The proliferation of smooth muscle cells plays crucial roles in AS pathogenesis. At the scale of ultramicroscopic structures, low density lipoprotein (LDL) continuously rises. The proliferation of smooth muscle cells is caused by the stimuli of growth factors released from damaged endothelial cells and malfunctional platelets. The major cellular component within the plaque is proliferated smooth muscle cells [3]. Therefore, the inhibition of smooth muscle cell proliferation is critical in controlling the progression of AS.

Recent studies found the crucial role of microRNA (miR) in the onset and progression of multiple diseases [4]. MiR is a group of non-coding small molecule single stranded RNA with a length of 18~24 bp, which can completely or incompletely bind with the 3'-untranslated regions (3'-UTR) of target gene mRNA. Under the direction of RNA exonuclease, it can selectively degrade target mRNA, thus inhibiting or activating downstream genes [5]. MiRs play important roles in basic cellular processes including proliferation, apoptosis, differentiation, and migration [6]. MiR-21 is a newly discovered miR and is related to cell proliferation by regulating PTEN. A previous study showed that a miR-21 inhibitor could target PTEN and suppress the proliferation of gastric cancer cells

[7], but this leaves its role in the proliferation of smooth muscle cells in AS unclear.

Serine/threonine protein kinase B (PKB/Akt) is an oncogene and can phosphorylate its downstream molecules and induce or regulate various biological activities including the cell cycle, cell growth, apoptosis and proliferation, thus participating in tumor onset and progression [3, 8]. The PI3K-Akt signal pathway is one of the most widely investigated signaling pathways in drug development and in the basic research fields [9]. A previous study showed that miR-21 could regulate neural stem cell proliferation by mediating Akt [10]. The role of miR-21 in regulating smooth muscle cell proliferation, however, is still unknown.

LDLr-/-mice are genetically engineered , homozygous mice with an extremely high serum cholesterol level which can present AS with a high fat diet (HFD) with a lipoprotein spectrum similar to humans. This study used HFD feeding LDLr-/-mice to generate an AS mouse model, from which the miR-21 expression profile and its role in AS were investigated, along with the effect of miR-21 on the AKT signaling pathway.

Materials and methods

Major reagents

A human aorta smooth muscle cell line (SMCs) was purchased from Cell Bank, Chinese Academy of Science (China). DMEM-F12 medium, penicillin, streptomycin, fetal bovine serum (FBS) and PBS buffer were purchased from Hyclone (US). POLDdeliver3000 and Opti-MEM medium were purchased from Invitrogen (US). A beta-actin endogenous reference antibody was purchased from Kangcheng Bio (China). PTEN, AKT and p-AKT antibody were purchased from Abcam (China). Rabbit anti-mouse IgG (H+L), rabbit anti-mouse IgG (H+L) were purchased from Proteintech (China). SYBR Green PCR Master Mix was purchased from Toyobo (China). An MiR-21 inhibitor and a negative controlled NC sequence were purchased from Gimma Gene (China).

Major equipment

The gel imaging system UVP Multispectral Imaging System (US) and the PS-9 semi-dry transferring electrophoresis were purchased from Jingmai (China). A Thermo-354 microplate reader was purchased from Thermo Fisher (US).

Experimental animals

C57 background LDLr-/- mice (6-8 weeks, body weight 18~22 g, N=60) were purchased from the Laboratory Animal Center of Shandong University (China). The mice were kept in clean facilities, with the temperature maintained at 24°C and the relative humidity at 60%. The mice received 12 h of light per day, with food and water ad libitum. Their bedding was changed daily to avoid infection.

The mice were used for all experiments, and all the procedures were approved by the Animal Ethics Committee of the Affiliated Hospital of Jining Medical University.

Animal model preparation

The laboratory animals were randomly divided into four groups: the normal control group, the AS model group, the antagomir-NC control group, and the antimir-21 experimental group. The normal control group was fed normal food, but the other three groups received an HFD for 16 weeks. The antagomir-NC control group and the antagomir-21 experimental group mice received a 50 ng/kg injection of antagomir-NC or antagomir-21 per week via a tail vein injection from the 10^{th} week of HFD feeding until the endpoint. The animals were then sacrificed and HE staining or oil-red O staining was performed to determine whether the model was established successfully.

HE staining

Mouse aorta tissues were harvested, fixed in 4% paraformaldehyde for 24 h, and dehydrated using 100%, 95%, 85%, and 75% gradient ethanol. The tissues were permeabilized and embedded in paraffin. 4 μm slices were prepared, de-waxed, re-hydrated and stained in hematoxylin. The slices were rinsed in tap water, differentiated in 1% HCl-ethanol, re-stained in eosin, rinsed in tap water, dehydrated in gradient ethanol, permeabilized, and mounted with coverslips.

Oil-red O staining

The aorta arch and the abdominal aorta were longitudinally dissected and fixed using nee-

dles. After PBS rinsing, the tissues were fixed in 60% isopropanol for 5 min, followed by oil-red O staining for 15 min. The damage area was presented by the ratio of the oil red O positive staining area to the total surface area.

Real time quantitative PCR

The aorta tissues or the cultured cells were mixed with 1 mL Trizol. After incubation on ice for 5 min, the tissues were mixed, and the lysates were removed into 1.5 mL EP tubes, followed by the addition of 0.2 mL chloroform. With 15 s of vigorous shaking, the tissue lysate was incubated at room temperature for 3 min. After 4°C centrifugation at 12000 g for 15 min, the upper aqueous phase was carefully removed to new EP tubes, which were mixed with 0.5 mL isopropanol. The mixture was incubated at room temperature for 10 min, and centrifuged at 12000 g for 10 min at 4°C. After discarding the supernatant, 1 mL ethanol was added and then the solution was washed three times. The supernatant was carefully removed, and 20 μL DEPC water was added to obtain an mRNA solution.

The MiR-21 primers were synthesized by Sigma (US) [11]. The sequences were: forward: 5'-TTGAA TTCTA ACACC TTCGT GGCTA CAGAG-3'; reverse: 5'-TTAGA TCTCA TTTAT CGAGG GAAGG ATTG-3'. U6 was used as the internal reference, forward: 5'-CTCGC TTCGG CAGCA CA-3'; reverse: 5'-AACGC TTCAC GAATT TGCGT-3' [12]. The reverse transcription was performed in a 20 μL system following the instructions of the assay kit. The PCR was performed in a 50 μL system following the instructions, with the conditions set as follows: 50°C for 30 min and 95°C for 5 min, followed by 40 cycles each consisting of 95°C for 30 s, 55°C for 30 s, and 72°C for 50 s, and it ended with a 72°C elongation for 5 min. After the reaction, the amplification and melting curves were confirmed. The Ct values of the target gene and internal reference gene were used for calculating the relative expression of genes using the $2^{-\Delta\Delta Ct}$ approach.

Western blot

Total protein solutions were quantified using the BCA approach and unified to unit concentration. The protein samples were mixed with a loading buffer and denatured in boiling water for 5 min. The samples were separated using 10% SDS-PAGE until the separation of the target protein from the adjacent proteins with a similar molecular weight was finished. The proteins were transferred to a PVDF membrane using a 300 mA current for 1 h. The membrane was incubated with antibodies against Akt or a p-Akt antibody (1:1000) overnight at 4°C. After rinsing with TTBS three times, a secondary antibody (1:1000) was added for a 37°C, 2 h incubation. The chemiluminescence method was applied to visualize the protein bands.

Cultures of the aorta smooth muscle cells

The aorta smooth muscle tissues were cultured in a DMEM medium containing 10% FBS and 1% dual antibiotics. The tissues were cultured in a 37°C chamber with 5% CO_2.

Using a previously documented approach [13], oxLDL (100DLNE) was used to treat the aorta smooth muscle cells to generate the AS model for smooth muscle proliferation.

Cell transfection

24 h before transfection, the aorta smooth muscle cells were inoculated into a 6-well plate. 10 pmol miR-21 antagomir or a respective negative control NC sequence, plus 5 μL POLD-deliver3000 were diluted into 100 μL for a 5 min mixing. Those mixtures were added into the cells in 6-well plate for a gentle mixture. After transfection, the cells were cultured for 48 h, and the miR or protein expressions were quantified.

MTT assay

After transfecting with an miR-21 antagomir, the cells at the log-growth phase were collected and adjusted to a density of $5\sim10\times10^4$/mL. A 100 μL cell suspension was added into each well of a96-well plate, in which 10 replicated wells were adopted for each group. After 24 h incubation, the cells were allowed to grow for 24 h continuous incubation. A 10 μL MTT solution was added, and the supernatant was carefully removed after 4 h incubation. A triple reaction solution was added (100 μL per well) for the mixture to completely resolve the crystal, followed by 12~15 h incubation. OD values at the 450 nm wavelength were measured. Cell proliferation efficiency was calculated using the normal group as 100%.

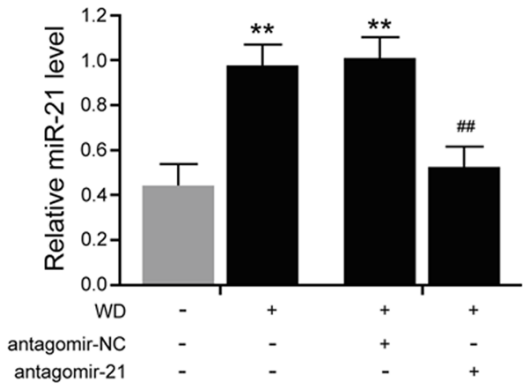

Figure 1. The expression levels of miR-21 in AS mouse aorta tissues. The aorta tissues were extracted from AS mice after different treatment followed by the isolation of total RNA, which was used for the analysis of miR-21 expression by quantitative PCR. **, $P < 0.05$ compared to the normal control group, ##, $P < 0.05$ compared to the western diet (WD) group.

Dual luciferase reporter gene assay

The liposome transfection app-roach was used to cotransfect miR-21 or the controlled NC oligonucleotides, plus the luciferase labelled plasmids (pIS0-PTEN-3'UTR-mut or pIS0-PTEN-3'UTR) or controlled plasmid into aorta smooth musclecells. A dual luciferase reporter gene assay kit was used to measure the luciferase activity following the instruction manual.

Statistical method

SPSS18.0 software was used to process all the experimental data, which were presented as the mean ± the standard deviation (SD). One-way analysis of variance (ANOVA) wase ployed for the comparison between groups. A further paired comparison was performed by a SNK-Q test. $P < 0.05$ indicated a statistical significance.

Results

MiR-21 expression level in the aorta tissues of AS mice

In the mouse AS model, the miR-21 expression level in the aorta tissues was significantly elevated ($P < 0.05$ compared to the control group). Antagmir-NC treatment did not significantly change miR-21 levels compared to the model group. However, antagmir-21 transfection remarkably inhibited the miR-21 expression level ($P < 0.05$ compared to model group, **Figure 1**).

Effect of miR-21 antagomir on aorta plaque formation

In the abovementioned study, we found significantly elevated miR-21 expressions in the AS model mice. Oil-red O staining on the mouse aorta tissues showed a significantly increased AS plaque lesion area in the model group. Antagmir-NC did not improve the condition of the artery plaque area, while antagmir-21 treatment significantly decreased the plaque area, as shown in **Figure 2**.

Moreover, we performed H&E staining to observe the morphology of aorta smooth muscle cells and AS plaque. As shown in **Figure 3**, we observed the formation of AS plaque and the migration and proliferation of smooth muscle cells in the AS model mice. Antagomir-NC treatment did not significantly change the aorta plaque area or the migration/proliferation of smooth muscle cells. Consistent with our expectations, antago mir-21 transfection significantly decreased the plaque area, and promoted the migration and proliferation of smooth muscle cells to a certain extent.

The expression level of miR-21 in aorta smooth muscle tissues

To further explore the effect of miR-21 on smooth muscle cell proliferation and migration, we measured the miR-21 expression in a smooth muscle tissue model treated with oxLDL. As shown in **Figure 4**, the miR-21 expression was significantly elevated in the smooth muscle cells after oxLDL treatment ($P < 0.05$), consistent with the findings from animal models.

Effects of miR-21 on smooth muscle cell proliferation

Within the smooth muscle cells treated by oxLDL, we measured the effect of miR-21 on cell proliferation via antagomir-21 transfection using an MTT assay. As shown in **Figure 5**, the cell proliferation activity was significantly enhanced in the oxLDL-treated smooth muscle cells ($P < 0.05$ comparing to control group). The treatment using antagomir-21 significantly suppressed cell proliferation ($P < 0.05$), demon-

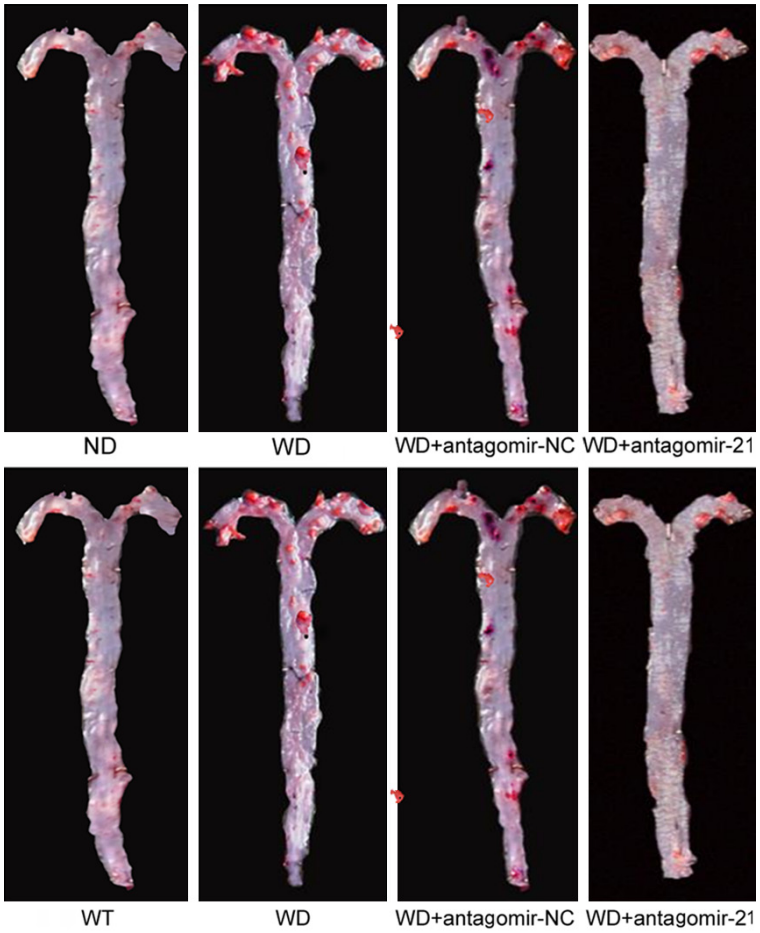

Figure 2. Oil-red O staining. ND, normal diet; WD, western diet. The aorta arch and the abdominal aorta were extracted from the AD mice after different treatment followed by being longitudinally dissected, fixed in 60% isopropanol, and subsequent oil-red O staining. The damaged area was presented by the ratio of oil red O positive staining area to the total surface area.

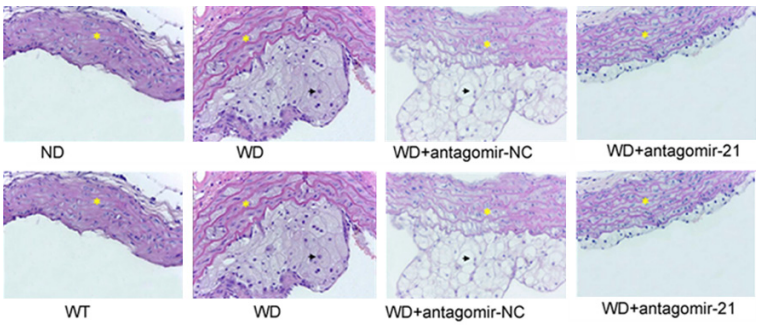

Figure 3. HE staining of aorta valves. ND, normal diet; WD, western diet. Mouse aorta tissues were fixed and dehydrated, followed by being permeabilized and embedded in paraffin. After that, 4 μm slices were prepared, de-waxed, re-hydrated and stained in hematoxylin and then re-stained in eosin followed by being mounted with coverslips.

strating that miR-21 played a role in smooth muscle cell proliferation.

Regulatory effects of miR-21 on PTEN expression

With reference to a database on targetscan and miRNA, PTEN was identified as target gene of miR-21. A previous study showed that PTEN expression could regulate the Akt signal pathway during cell proliferation, but its role in AS smooth muscle cell proliferation has not been investigated yet. As shown in **Figure 6A**, we constructed a luciferase reporter gene plasmid carrying wild type (WT) and mutant (MT) forms of PTEN, in order to investigate the relationship between miR-21 and PTEN. The results of luciferase reporter gene are shown in **Figure 6B**. After transfecting miR-21, the luciferase activity of WT form of PTEN was significantly suppressed, but no change of luciferase activity was found in the mutant form of PTEN. These results showed that miR-21 targeted PTEN in smooth muscle cells. Moreover, we measured the effect of antagomir-21 on PTEN expression in oxLDL-treated smooth muscle cells. As shown in **Figure 6C**, PTEN expression was significantly decreased in the oxLDL-treated smooth muscle cells ($P < 0.05$ compared to the control group). However, the transfection of antagomir-21 significantly increased the PTEN expression ($P < 0.05$). These results proved that miR-21 could target and inhibit PTEN expression.

PI3K/Akt signal pathway participates in miR-21 regulated cell proliferation

The PI3K/Akt signal pathway is a classic pathway regulating cell proliferation. A previous study showed that PTEN inhibited PI3K/Akt signal pathway activa-

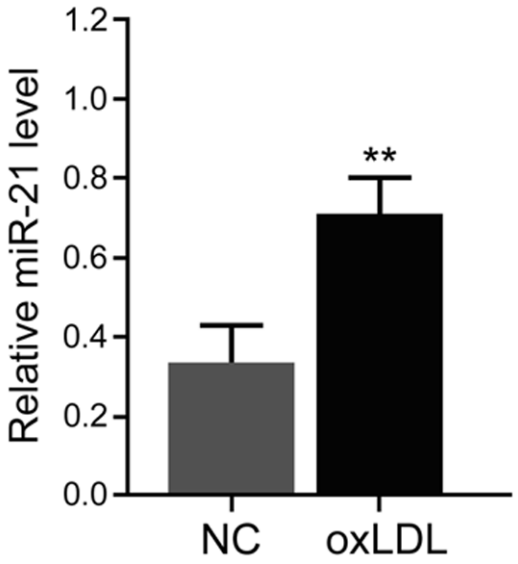

Figure 4. The expression of miR-21 in aorta smooth muscle cells. Aorta smooth muscle cells were cultured and collected followed by RNA isolation for the measurement of miR-21 expression by quantitative PCR. **, P<0.05 compared to NC group.

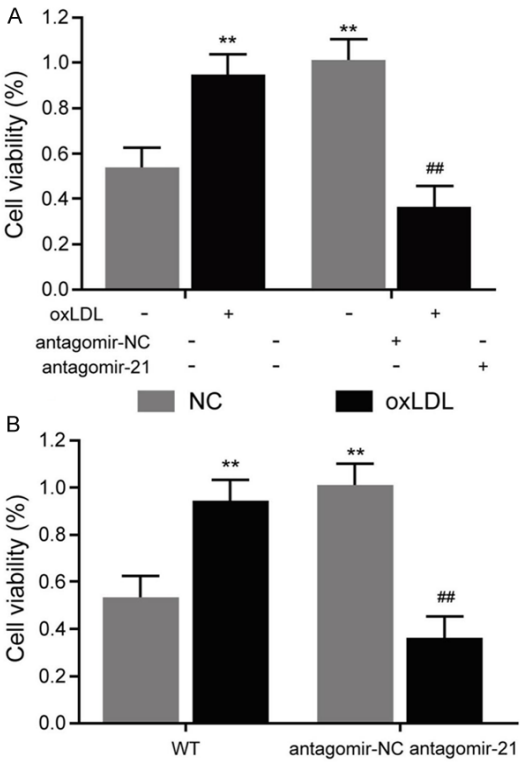

Figure 5. The effects of miR-21 on smooth muscle cell proliferation. Cultured aorta smooth muscle cells (A) or the AS mouse model (B) followed by measuring cell proliferation using an MTT assay. **, P<0.05 compared to NC group; ##, P<0.05 compared to WT group (oxLDL).

tion, but miR-21 targeted and inhibited PTEN. We thus treated the cells with antagomir-21 to measure the Akt signal pathway activity. As shown in **Figure 7**, the oxLDL treatment remarkably activated the Akt signal pathway, and enhanced the phosphorylation of Akt ($P < 0.05$ compared to normal control group). These results were consistent with the enhanced proliferation of smooth muscle cells. In contrast, antagomir-21 treatment inhibited the phosphorylation of Akt ($P < 0.05$ comparing to oxLDL group). These results demonstrated the association between miR-21-induced cell proliferation and the Akt signaling pathway transduction.

Discussion

AS is a disease characterized by the thickening and rigidity of the arterial wall, which presents plaque-like patterns [14]. The AS plaque is the major factor causing the thickening and rigidity of arterial walls, and it is a critical step in AS pathogenesis. With an improved quality of life and a transition of lifestyles, the incidence of AS is rapidly increasing. Therefore, research on the pathogenic mechanisms of AS is of critical importance.

Recent studies showed differential expressions of miRNA during AS pathogenesis [15], indicating that miRNA might be involved in AS occurrence. A retrospective study indicated a correlation between miRNA expression and the instability of AS plaque in animal models and human studies, and found that miR-21, miR-100, miR-127, miR-133, and miR-143 participate in the onset and progression of AS [15-17]. A dynamic change in miR-21 expression has been the focus of recent AS studies. In oxLDL-induced aorta endothelial cells, the inhibition of miR-21 expression can suppress endothelial cell injury by facilitating cell autophagy [18]. Another study showed that long noncoding RNA (lncRNA) MEG3 could mediate endothelial cell proliferation and migration by modulating miR-21 [19]. However, in macrophages within the AS plaque, a reduced miR-21 expression can facilitate cell apoptosis, plaque necrosis, and vascular inflammation [20]. Although the expressional profile of miR-21 in various cells during AS pathogenesis is inconsistent, all the studies proved the important role of miR-21 in AS pathogenesis. Currently no study has been performed regarding the expres-

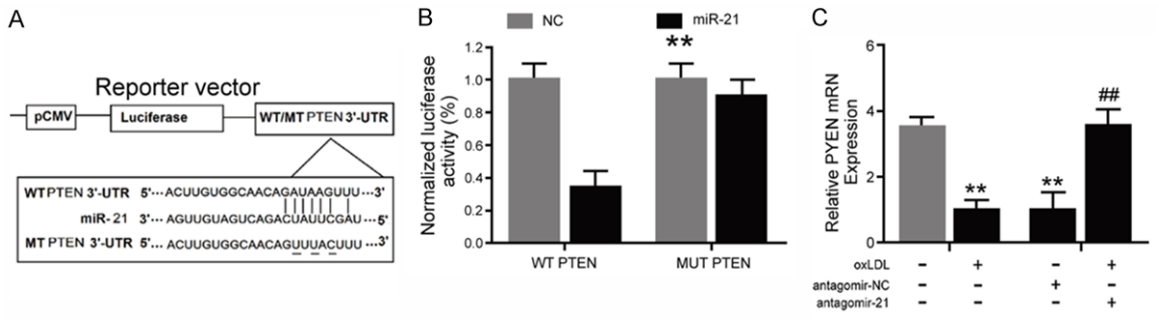

Figure 6. MiR-21 targeted and inhibited PTEN expression. Liposome was used to co-transfect miR-21 or controlled NC oligonucleotide, plus luciferase labelled plasmids (pISO-PTEN-3'UTR-mut or pISO-PTEN-3'UTR) or controlled plasmid into aorta smooth muscle cells (A) followed by an analysis of relative luciferase activity (B) and PTEN mRNA level (C). **, P<0.05 compared to the normal control group. ##, P<0.05 compared to the oxLDL group.

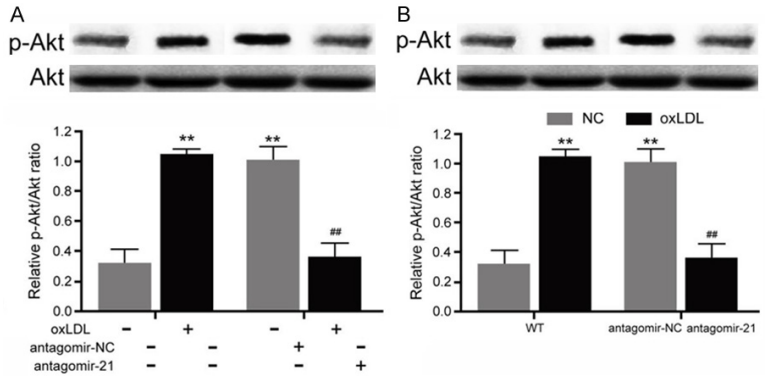

Figure 7. The expression levels of Akt and p-Akt. Cultured aorta smooth muscle cells (A) or the AS mouse model (B) were treated with antagomir-NC or antagomir-21 followed by measuring the expression of AKT and p-AKT. **, P<0.05 compared to the normal control group. ##, P<0.05 compared to the oxLDL group.

sion of miR-21 in smooth muscle cells, nor its effect on cell proliferation. We thus investigated the effect of miR-21 on aorta plaque formation and the proliferation of smooth muscle cells, along with the potential mechanisms. We first identified a significant increase of miR-21 expression in the AS mouse model, consistent with the previous findings in endothelial cells [21].

With reference to databases such as targetscan and miRNA, we found the targeted regulation of PTEN by miR-21 [22], and PTEN had an inhibitory role on the PI3K/Akt signal pathway [23]. So, whether miR-21 participates in the process of AS smooth muscle proliferation has attracted our research interest.

Located in chromosome 10q22.3, the PTEN gene codes a protein consisting of 403 amino acids with phospholipase activity. Early studies in tumor biology showed that the PTEN protein could inhibit tumor onset and progression by antagonizing the activity of phosphatase such as tyrosine kinase [24]. Other studies showed the indispensable role of PTEN in cell growth, and the inhibitory effect on the Akt signal was related to the removal of the 3rd phosphate group of IP3 [25]. In this study, we found miR-21 over-expression in AS model, and targeted the degradation of PTENmRNA, thus relieving the inhibitory effects on the PI3K/Akt signal pathway by PTEN for facilitating cell proliferation. These findings are consistent with the previous findings regarding tumor cells Meanwhile, we used antagomir-21 to specifically inhibit miR-21 expression at both the animal and cell levels and obtained the opposite results to those under miR-21 overexpression.

This study for the first time measured the upregulation of miR-21 in AS smooth muscle cells, and its role in targeting PTEN to activate the AKT signaling pathway for facilitating smooth muscle cell proliferation, indicating that miR-21 might work as a target for treating AS. However, due to the differential expression of miR-21 across related cells during AS pathogenesis, its application is largely restricted. In future studies, it is necessary to examine the expressional pattern of miR-21 in various cells during AS onset, and the underlying mechanisms.

Conclusion

MiR-21 targets and inhibits PTEN to activate the AKT signal pathway, leading to the enha nced proliferation of smooth muscle cells, and the increased formation of AS plaque. Downregulating miR-21 expression might work as a potential therapeutic approach for treating and preventing AS.

Disclosure of conflict of interest

None.

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