MiR-182-5p Inhibits the Proliferation of Vascular Smooth Muscle Cells Induced by ox-LDL Through Targeting PAPPA

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Summary
This study aims to analyze the expression level and correlation of miR-182-5p and its target gene PAPPA in coronary atherosclerosis (CAD).

Real time PCR, ELISA, and Western blotting methods were used to detect the expression levels. Dual-luciferase reporter gene assays were used to analyze the interaction between the 3'-UTR of PAPPA and miR-182-5p.

The expression level of miR-182-5p in CAD was significantly lower than that in normal population, while the content of serum PAPPA was significantly increased, and the expression level of miR-182-5p was negatively correlated with the PAPPA content. The expression level of miR-182-5p decreased, while the expression level of PAPPA increased significantly in the ox-LDL treated HA-VSMC cells. Researchers found that PAPPA could promote the activation of IGF signaling pathway in HA-VSMC cells treated by ox-LDL, further activate NF-kB, PI3K/AKT and ERK signaling pathway, and promote cell proliferation. However, miR-182-5p could inhibit the expression of PAPPA, block the activation of IGF signal pathway, and inhibit the proliferation of HA-VSMC cells induced by ox-LDL. miR-182-5p had a targeted action site in the 3'-UTR of PAPPA by bioinformatics prediction. The analysis of luciferase reporter gene further confirmed that miR-182-5p could target the 3'-UTR of PAPPA to inhibit its expression.

miR-182-5p demonstrated a protective effect on atherosclerosis and may be a potential therapeutic target for atherosclerosis.

Key words: miRNAs, Atherosclerosis, RNA interference, Signaling pathway, qRT-PCR

Cardiovascular disease is one of the main causes of death in developed countries.17 Atherosclerosis (AS) is the most common cause of cardiovascular disease, and AS is significantly related to its morbidity and mortality.18 However, its pathogenesis has not been fully understood. As we all know, oxidized low-density lipoprotein (ox-LDL) is one of the main risk factors of AS.19 Ox-LDL can promote the formation of foam cells derived from vascular smooth muscle cells (VSMC) and regulate the proliferation, apoptosis, migration, and differentiation of VSMC, which play a crucial role in the pathogenesis of cardiovascular diseases.20 However, the detailed mechanism of how ox-LDL induces VSMC proliferation has not been fully understood.

MicroRNA (miRNA) is a kind of non-coding miRNA, the length is usually 18-25 nucleotides. miRNA can inhibit translation and cut mRNA by pairing with the 3' untranslated base of the target gene. They became the main regulators of various biological and pathological processes, including cell differentiation, apoptosis, and proliferation. More and more evidences showed that miRNA played an important role in cardiovascular system.21-27 Researchers confirmed that miR-21, miR-221, miR-222, and miR-145 played a regulatory role in the proliferation of VSMC, which was the key pathological process of atherosclerosis.28-32 Platelet-derived growth factor mediated podosome formation in SMCs through the regulation of miR-143/145 expression via a pathway involving Src and p53, and miR-143/145 gene products inhibited podosome formation in VSMCs.33 MiRNA also
played a key role in the pathogenesis of ox-LDL mediated inflammation.\textsuperscript{16} Ox-LDL could regulate the expression of let-7g, thus inhibiting the expression of LOX-1 and OCT-1 in human coronary artery smooth muscle cells.\textsuperscript{17} However, the role of miRNA in the proliferation of VSMC induced by ox-LDL remains to be further studied. Previous studies showed that the expression level of miR-182-5p in a variety of tumors decreased significantly, and upregulation of miR-182-5p could inhibit the proliferation of tumor cells.\textsuperscript{18,19} In addition, miR-182-5p could also inhibit inflammation, and miR-182-5p alleviated the nonalcoholic steatohepatitis induced by high-fat diet in mice.\textsuperscript{20} Since inflammation and VSMC abnormal proliferation were also involved in the pathogenesis of atherosclerosis, we speculated that miR-182-5p may be related to atherosclerosis and may play a role in the proliferation of VSMC induced by ox-LDL.

PAPPA was first found in pregnant women. It was produced by syncytiotrophoblast cells and was a tetramer. Researchers found that osteoblasts, fibroblasts, endothelial cells, VSMC, etc. can produce homodimers with proteolytic activity subsequently.\textsuperscript{21} Study showed that PAPPA was a potential biomarker of plaque instability and inflammation in patients with acute coronary syndrome.\textsuperscript{22} Increased PAPPA levels were associated with an increased load of 3-vascular thin-walled fibrous atheromatous plaques (TCFA) in patients with coronary artery disease.\textsuperscript{23} In this study, we analyzed the expression level and correlation of miR-182-5p and its target gene PAPPA in coronary atherosclerosis (CAD).

Methods

Samples: The peripheral blood of patients with CAD was collected from the Department of vascular medicine, the First Affiliated Hospital of Anhui Medical University from January to December 2018, and the peripheral blood of normal physical examination population was taken from the physical examination center of the First Affiliated Hospital of Anhui Medical University from January to December 2018. All the selected patients with CAD were confirmed by coronary angiography (lumen stenosis > 50%). Exclusion criteria were as follows: cardiac arrest, cardiogenic shock, hypotension or pulmonary congestion, those patients with malignant tumor, chronic inflammatory disease (inflammatory bowel disease, rheumatoid arthritis, and systemic lupus erythematosus), infection, and patients using glucocorticoids.

Cell culture and small interfering (si) RNA transfection: Human aortic smooth muscle cells (HA-VSMC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured with F-12K medium (ATCC, Manassas, VA, USA) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin / streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37°C in 5% CO₂. The cells in the ox-LDL group were treated with 0, 10, 20, 40, 60, and 80 µg/mL of ox-LDL (Sigma-Aldrich, St. Louis, MO, USA) for 48 hours. The mimics, overexpression, and siRNA vectors of miR-182-5p or PAPPA were transfected into HAECs, respectively, with Lipofectamine 3000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manual.

Enzyme-linked immunosorbent assay (ELISA): The PAPPA level in peripheral blood was detected by ELISA Kit (Abcam, Cambridge, UK). The specific operation was carried out according to the instructions. OD450 value was measured by Microplate spectrophotometer.

Cell proliferation assay: The cell proliferation was detected by Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manual protocol. The cells in the logarithmic growth phase were digested with trypsin and inoculated into 96-well plates (5,000 cell/well), and they were cultured at 37°C with 5% CO₂ overnight. To each well was added 10 µl cck-8 solution before detection and incubation for 1 hour, and A450 values were detected by Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA) after culture for 24 hours, 48 hours, 72 hours, and 96 hours, respectively, to evaluate the proliferation of cells.

DNA extraction and qRT-PCR: Total RNA were extracted by using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol. RNA concentration and purity were detected using a QuantiChrom RNA Assay Kit (BioAssay Systems, Inc.). A total of 1 µg RNA was subjected to reverse transcription using a Prime Script II kit (Takara Bio Inc.). The expression of miR-182-5p was detected by miRNA specific reverse transcription kit (D350, Takara, Tokyo, Japan) and then analyzed by qPCR with Power SYBR Green (DRR081A, Takara, Tokyo, Japan). The quantification method used was the 2⁻∆∆CT method. The thermocycling conditions were as follows: pre-degeneration at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 62°C for 10 sec, and U6 and β-actin genes were used as an internal control. The primers used in this study are shown in the Table.

Western blotting detection: Cells in different groups were harvested and lysed with cell lysis solution (Sigma-Aldrich, St. Louis, MO, USA). The supernatant was collected after they were centrifuged at 4°C (1000 rpm) for 5 minutes. Total proteins were extracted, and protein concentration was determined using BCA. Proteins (50 µg per lane) were separated using 12% SDS-PAGE. Then, proteins were electrotransferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The PVDF membrane was rinsed with TBS for 10-15 minutes and placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder. It was incubated at room temperature for 2 hours, following the addition of an appropriate dilution of primary antibodies (1:1000 PAPPA; 1:5000 GAPDH; 1:500 IGFBP4; 1:2000 NF-kB-p65; 1:10000 AKT; 1:500 p-AKT; 1:1000 ERK1/2; 1:400 p-ERK1/2; 1:1000 H3; Abcam, Cambridge, UK). Then, the membrane was rinsed with TBST three times (5-10 minutes/wash) and then incubated at room temperature for 1 hour with horseradish peroxidase-labeled secondary antibody (1:10,000; Abcam, Cambridge, UK; diluted with TBST containing 0.05% (w/v) skimmed milk powder). Then, the membrane was rinsed three times with TBST (5-10 minutes/wash). Protein bands were detected using an en-
hanced chemiluminescence kit (Perkin-Elmer Inc.) and quantified as the ratio to GAPDH. Quantification was performed using Imagequant LAS4000 (GE Healthcare, Japan).

**Dual-luciferase reporter gene assays:** The luciferase reporter gene plasmid PAPPA 3’-UTR (pGL3-PAPPA-UTR-WT) and point mutation of luciferase reporter gene plasmid PAPPA 3’-UTR (pGL3-PAPPA-UTR-Mut) were constructed to analyze the interaction between the 3’-UTR of PAPPA and miR-182-5p. The HA-VSMC cells were inoculated into 24 well plates and cultured overnight, and luciferase reporter plasmid, Renilla luciferase and miR-182-5p mimic or control were transfected into HA-VSMC cells simultaneously. The cells were split using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA), according to the manual after culture for 48 hours. The results were detected by Panomics Lumimeter (Affymetrix, Santa Clara, CA, USA), after the luminescence was added. The sea renin fluorescence was used as internal reference.

**Statistical analysis:** The data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation (SD). Student’s *t* test, the Wilcoxon signed-rank test or Pearson’s chi-square test were used to evaluate the differences among groups. The correlation between PAPPA and miR-182-5p were analyzed by Pearson’s correlation analysis. *P* < 0.05 was considered to be significant.

**Results**

The expression of miR-182-5p in peripheral blood of patients with CAD decreased, while the expression of PAPPA increased: RT-PCR results showed that the expression level of miR-182-5p in the peripheral blood of patients with CAD (*n* = 50) was significantly lower than that of the control group (*n* = 50, Figure 1A, *P* < 0.01), while the expression level of PAPPA was significantly higher than that of the control group (Figure 1B, *P* < 0.01). The correlation between the expression level of miR-182-5p and PAPPA showed they demonstrated a contrary trend (Figure 1C).

**ox-LDL inhibited the expression of miR-182-5p and promoted the expression of PAPPA in HA-VSMC:** Cell proliferation analysis showed that ox-LDL could promote the proliferation of HA-VSMC (Figure 2A, *P* < 0.05), inhibit the expression of miR-182-5p (Figure 2B, *P* < 0.01), and promote the expression of PAPPA with dose-dependent (Figure 2C, *P* < 0.01). The effect was the most obvious when the HA-VSMC was treated by 40 μg/mL ox-LDL (*P* < 0.01). Overexpression of PAPPA in normal VSMCs demonstrated no significant effect on the expression level of miR-182-5p (Data not shown).

**ox-LDL could activate NF-kB p65, PI3K/AKT and ERK signaling pathway by promoting PAPPA expression and inhibiting IGFBP4 expression:** In the HA-VSMC treated with ox-LDL, the expression level of PAPPA, p-AKT, and p-ERK1/2 increased, the expression level of IGFBP4 decreased, and the level of NF-kB p65 nuclear translocation increased (Figure 3). These results suggested that ox-LDL could activate the NF-kB, PI3K/AKT, and ERK signaling pathway by promoting PAPPA expression. When IGFBP4 was transfected in the PAPPA overexpressed HA-VSMC cells at the same time, the expression of IGFBP4 was significantly increased and significantly inhibited cell proliferation (Data not shown).

**miR-182-5p inhibited the expression of PAPPA and the proliferation of HA-VSMC treated with ox-LDL:** miR-182-5p siRNA promoted the proliferation of HA-VSMC, while miR-182-5p Mimics inhibited the proliferation of HA-VSMC. miR-182-5p could inhibit the expression of PAPPA mRNA and protein and the activation of NF-kB, PI3K/AKT, and ERK signaling pathway (Figure 4).

**Down regulation of PAPPA expression inhibited the proliferation of HA-VSMC treated by ox-LDL:** Overexpression of PAPPA promoted the proliferation of HA-VSMC treated by ox-LDL, while PAPPA siRNA inhibited the proliferation of HA-VSMC. PAPPA siRNA could not inhibit the activation of the NF-kB, PI3K/AKT, and ERK signaling pathway. However, PAPPA siRNA could not inhibit the miR-182-5p expression (Figure 5).

**miR-182-5p could target 3’-UTR of PAPPA to inhibit its expression:** We predicted that a binding site existed between miR-182-5p and the 3’-UTR of PAPPA, by three kinds of software analysis (PicTar, http://pictar.bio.nyu.edu/u/; TargetScan7.1, http://www.targetscan.org/ and miRanda, http://www.microrna.org/). PCR and WB results showed that miR-182-5p regulated the PAPPA expression directly. The results of double luciferase reporter gene also confirmed that miR-182-5p could target the 3’-UTR of PAPPA. When the point mutation was carried out in the 3’-UTR prediction region of PAPPA, the activity of luciferase was no longer regulated by miR-182-5p (Figure 6).

**Table.** Primers Used in This Study

| Primers  | Sequences (5’-3’)              |
|----------|-------------------------------|
| PAPPA    | F: ACAAAGACCCACGCTACTTTTTR: CAGAACTGCCCATCATAAGGTG |
| miR-182-5p| GTCGATACAGTCAGGTCGCCAGGTCGCCAGGTCGTTGGAAGGTATTCGCACTGGATACGACAGTG |
| U6       | F: CTGCCTCCGGACGCARR: AACGCTTCAGGAATTTGCGT |
| β-actin  | F: TGAGGATGTCACGTTCCAGR: GTCACCCTTCACCGTTCCAGT |
PROTECTIVE EFFECT OF miR-182-5p ON ATHEROSCLEROSIS

Figure 1. The expression level of miR-182-5p and PAPPA in the peripheral blood of patients with CAD. A: miR-182-5p expression level; B: PAPPA levels; C: Correlation analysis of the expression level of miR-182-5p and PAPPA.

Figure 2. Effects of ox-LDL on the proliferation of HA-VSMC and the expression of miR-182-5p and PAPPA. A: Effect of ox-LDL on the proliferation of HA-VSMC; B: Effect of ox-LDL on the expression of miR-182-5p in HA-VSMC; C: Effect of ox-LDL on the expression of PAPPA mRNA in HA-VSMC. (n = 3, *P < 0.05)
Discussion

Researchers confirmed that ox-LDL is the main risk factor of atherosclerosis. In the early stage of atherosclerosis, ox-LDL infiltrates into the subendothelial layer through injured endothelial cells, causing a series of pathological events. As we all know, the proliferation of VSMC is a key event in the pathogenesis of atherosclerosis. ox-LDL plays a key role in the development of atherosclerosis by stimulating the proliferation of VSMC,
Figure 5. Effects of PAPPA siRNA or overexpression on the proliferation of HA-VSMC treated with ox-LDL. A: CCK-8 detection results; B: miR-182-5p expression levels; C: PAPPA mRNA expression levels; D: WB detection of PAPPA and IGFBP4 expression levels in HA-VSMC; E: Detection of NF-kB p65 activation in HA-VSMC; F: Detection of the activation of AKT and ERK1/2 signaling pathway in HA-VSMC. (n = 3, *P < 0.05, **P < 0.01)

Figure 6. miR-182-5p could target 3′-UTR of PAPPA to inhibit its expression. A: Software analysis predicted that there was a binding site between miR-182-5p and the 3′-UTR of PAPPA; B: Effects of miR-182-5p on PAPPA mRNA expression; C: Luciferase reporter gene analysis showed that miR-182-5p could target the 3′-UTR of PAPPA; D: Effects of miR-182-5p on PAPPA protein expression. (n = 3, **P < 0.01)
but its specific molecular mechanism was not fully defined.

In this study, we found that the expression level of miR-182-5p decreased, while the expression level of PAPPA increased in patients with CAD, and a correlation was found between them. Treatment of HA-VSMC with ox-LDL in vitro could inhibit the expression of miR-182-5p and promote the expression of PAPPA, and PAPPA further cut the IGFBP4 and activated IGF signaling pathway, which resulted in activation of the NF-kB, PI3K/AKT, and ERK signaling pathway and abnormal proliferation of HA-VSMC. miR-182-5p could target 3'-UTR of PAPPA to inhibit its expression. These results suggested that ox-LDL may inhibit the expression of miR-182-5p in the process of atherosclerosis, and miR-182-5p may be a new potential treatment target for vascular diseases (Figure 7).

More and more studies revealed the role of microRNAs in the occurrence of AS. For example, the decrease of miR-21 in macrophages could lead to apoptosis, plaque necrosis, and vascular inflammation in AS, miR-155 could promote the autophagy of human umbilical vein endothelial cells induced by ox-LDL, and miR-9 could inhibit the activation of NLRP3 in AS inflammatory cell model by regulating JAK1/STAT signaling pathway.\textsuperscript{26-29} miR-182-5p could inhibit cisplatin induced apoptosis of inner ear hair cells by down regulating FOXO3a expression.\textsuperscript{30} The decreased expression of miR-182-5p could promote the progression of renal cancer by activating Akt/FOXO3a signaling pathway.\textsuperscript{31} miR-182-5p could inhibit the expression of MITF, BCL2, and cyclinD2 to inhibit the progression of ovarian cancer.\textsuperscript{32} In gastric cancer, miR-182-5p could also target cAMP responsive element binding protein 1 to inhibit tumor proliferation.\textsuperscript{33} The previous study also showed that the expression level of miR-182-5p decreased in RAW264.7 cells induced by ox-LDL, and up regulation of miR-182-5p expression could inhibit lipid accumulation in cells and prevent the progress of AS.\textsuperscript{34} In this study, we found that up regulation of miR-182-5p expression could inhibit abnormal proliferation of HA-VSMC cells, which suggested that miR-182-5p participated in the occurrence and development of AS.

PAPPA is a new zinc metalloproteinase, which is involved in the proteolysis of IGFBP-4 and IGFBP-5.\textsuperscript{35} When IGF is released from the IGFIGFBP complex, IGF combines with IGF-IR to rapidly increase the bioavailability of IGF and lead to the activation of many signal pathways.\textsuperscript{36} Therefore, PAPPA plays an important role in IGF signal transduction. The study showed that PAPPA may be a marker of ACS, which could predict the clinical outcome. A positive correlation was found between increased PAPPA levels and cardiovascular events, and the positive correlation was not affected by follow-up time, type of coronary disease, and different measurements of PAPPA.\textsuperscript{37} Some animal model studies also showed that PAPPA may be involved in the development of atherosclerosis.\textsuperscript{22,37} CRP could induce the expression and secretion of PAPPA in human peripheral blood mononuclear cells through a NF-kB dependent pathway, which may play an important role in the elevation of serum PAPPA level in patients with ACS.\textsuperscript{38} In this study, we found that the level of PAPPA increased in the peripheral blood of patients with AS. The expression level of PAPPA increased, and the IGFBP-4 content decreased in human HA-VSMC treated by ox-LDL and NF-kb. PI3K/AKT and ERK signaling pathways were activated. Overexpression of miR-182-5p inhibited the expression of PAPPA, the activation of NF-kb, PI3K/AKT and ERK signaling pathway, and the proliferation of HA-VSMC. Therefore, the miR-182-5p/PAPPA signal axis may be one of the molecular mechanisms regulating the abnormal proliferation of VSMC in the process of atherosclerosis.

**Conclusions**

In conclusion, we found a correlation between miRNA expression and atherosclerosis in this study. miR-182-5p regulated the PAPPA expression and participated in the abnormal proliferation of VSMC in the process of atherosclerosis. Our results suggested that miR-182-5p demonstrated a protective effect on atherosclerosis, and its mechanism may be related to the inhibition of IGF signaling pathway induced by PAPPA and the activation of downstream NF-kB, PI3K/AKT and ERK, and the reduction of abnormal proliferation of VSMC. miR-182-5p may be a potential therapeutic target for atherosclerosis, but its function and clinical application need further study.

**Disclosure**

**Conflicts of interest:** The authors have no conflicts of in-
terest to declare.

Statement of ethics: This study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The written informed consent was obtained from patients. The patient consent was written informed consent. All the experiments were approved by the Ethics Committee of Anhui medical university.

Author contributions: Conceived and designed the experiments: Shan Gao; Execution of experiments: Chaolong Jin, Dayuan Li, Zhiheng Ding and Xuegong Shi; Data analysis: Zhangyue Hu, Chunmiao Wang, Jie Xiao, Zhe Sheng, Yue Xu and Zhiheng Ding; Discussion of results: Dingxin Zhang, Di Wang, Tingting Wang, Fang Yang, Ying Yang, Xinglong Wang and Liping Wu; Wrote and or critical reading of manuscript: Chaolong Jin and Shan Gao.

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