# Apoptosis in HUVECs induced by microRNA-616-3p via X-linked inhibitor of apoptosis protein targeting

HUA CHEN, XI LIU, YUN WU, XIAYIN WU, XIAOLI WEN, YANAN LU and XINGSHENG ZHAO

Department of Cardiology, Inner Mongolia People's Hospital, Hohhot, Inner Mongolia 010000, P.R. China

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**Abstract.** Atherosclerosis causes stroke and coronary heart disease and is associated with a high mortality rate worldwide. However, the pathogenesis of atherosclerosis remains unclear. Endothelial cell apoptosis is one of the early changes observed in atherosclerosis. Previous studies have found that microRNA (miR)-616-3p may be involved in the development of atherosclerosis, but the specific mechanism is not clear. The present study aimed to investigate whether miR-616-3p is involved in endothelial cell apoptosis and its underlying mechanism. The present study demonstrated that compared with normal HUVECs, HUVECs treated with oxidized low-density lipoprotein expressed higher miR-616-3p and lower X-linked inhibitor of apoptosis protein (XIAP) levels. In the present study, HUVECs were transfected with miR-616-3p mimic and Cell Counting Kit-8 (CCK-8), flow cytometry and TUNEL staining assays demonstrated that compared with miR-616-3p mimic control, the miR-616-3p mimic promoted HUVEC apoptosis. In addition, using StarBase 3.0 for bioinformatics analysis it was predicted that miR-616-3p may bind to the 3'untranslated region (UTR) of XIAP mRNA. The present study performed the CCK-8, flow cytometry, TUNEL staining and dual-luciferase reporter assays and demonstrated that miR-616-3p binds to the 3'UTR of the XIAP mRNA and inhibits its expression and that this further promotes apoptosis in HUVECs. In addition, western blotting demonstrated that compared with miR-616-3p mimic control, the miR-616-3p mimic increases the level of cleaved caspase-3 in HUVECs. In summary, the present study demonstrated that miR-616-3p can directly inhibit the expression of XIAP mRNA by targeting its 3'UTR which promoted apoptosis in HUVECs. miR-616-3p and XIAP may be used as therapeutic targets of atherosclerosis in the future.

*Correspondence to:* Dr Xingsheng Zhao, Department of Cardiology, Inner Mongolia People's Hospital, 20 Zhaowuda Road, Hohhot, Inner Mongolia 010000, P.R. China
E-mail: 2182228287@qq.com

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## Introduction

Atherosclerosis is a disease in which atherosclerotic plaques are deposited in arterial wall and cause arterial stenosis (1). Atherosclerosis may lead to coronary artery disease, stroke and peripheral artery disease (2). Globally, 7.4 million deaths are caused by coronary heart disease each year and 6.7 million patients die from stroke in 2015 (3). The exact mechanism of atherosclerosis is still not fully understood, but endothelial cell apoptosis is known to be one of the important mechanisms underlying atherosclerosis progression (4).

Endothelial cell injury, which is one of the earliest pathophysiological changes in atherosclerosis promotes the production of inflammatory mediators, such as interleukin-1β (5) and free radicals including reactive oxygen species and reactive nitrogen species (6) to form an inflammatory and an oxidative stress environment (7). Normal endothelium serves a role in regulating vascular tone, cell adhesion, smooth muscle cell proliferation and in maintaining vascular homeostasis (8). A previous have found a significant increase in endothelial cell apoptosis in atherosclerotic blood vessels and plaques (9). Apoptosis of endothelial cells allows leukocytes and low-density lipoprotein (LDL) to pass through the blood vessel wall more easily and continuous accumulation of LDL in the endothelium, which results in plaque formation and subsequently, atherosclerosis (10). In accordance, some factors that cause atherosclerosis, such as high levels of LDL, elevated blood glucose levels, reduced nitric oxide levels and increased oxidative stress levels have been associated with an increase in endothelial cell apoptosis (11). Apoptosis of endothelial cells results in activation of the coagulation system, which is followed by destruction of the vascular endothelium and even, local thrombosis that can eventually lead to vascular occlusion, unstable angina pectoris, heart attack and stroke (12). Based on all these findings, a growing number of researchers have been focusing on how endothelial cell apoptosis can be inhibited.

MicroRNA (miR) is a ribonucleic acid molecule with a length of 21-23 nucleotides that is widely present in eukaryotes and can regulate the expression of other genes (13). Various miRNAs have been found to serve an important role in the pathogenesis of atherosclerosis. For example, miR-34 has a protective effect against oxidative stress in endothelial cells (14) and miR-155 has the ability to destroys tight junctions and the integrity of endothelial barriers, leading

to an increased endothelial permeability and enhanced atherosclerotic progression (15). In addition, miR-345-3p, miRNA-26a-5p, miR-142-3p were found to regulate endothelial cell apoptosis (16-19). Additionally, previous studies found that miR-616-3p may be involved in the development of coronary atherosclerosis. For example, miR-616-3p was found to participate in the development of atherosclerosis by directly acting on the 3'UTR of paraoxonase 1 (PON-1), but the specific mechanism is still unclear (20). Another study demonstrated that miR-616-3p single nucleotide polymorphisms at PON1 could affect genetic expression and that this was associated with an elevated risk for ischemic stroke and subclinical atherosclerosis (21). Hence, the precise role of miR-616-3p in the pathogenesis of coronary atherosclerosis is not clear (21). It may be beneficial to explore the mechanisms of miR-616-3p in endothelial cell injury, as it may prove to be a potential treatment target for coronary atherosclerosis in the future.

Based on all the previously reported findings, the present study hypothesized that miR-616-3p is involved in the pathogenesis of coronary atherosclerosis via its effect on endothelial cell apoptosis. In addition, the present study aimed to explore the potential mechanisms via which miR-616-3p may play a role in endothelial cell apoptosis. The purpose of this study was to elucidate the mechanism of atherosclerosis and to find new therapeutic targets for atherosclerosis. miR-616-3p and XIAP may be used as future therapeutic targets of atherosclerosis.

## Materials and methods

*Cell culture and treatment.* HUVECs (ATCC® PCS-100-013™) were purchased from ATCC and cultured in RPMI-1640 medium (Thermo Fisher Scientific Inc.) containing 10% FBS (Hyclone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin solution. The cells were cultured at 37˚C in a humidified incubator containing 5% $CO_2$ and passaged when they reached 90% confluence. Subsequently, HUVECs were planted in 6-well plates at a density of $1x10^6$ per well and were treated with RPMI-1640 medium (with 10% FBS and 1% penicillin-streptomycin solution) containing 60 µg/ml oxidized low-density lipoprotein (ox-LDL) for 48 h at 37˚C in a humidified incubator containing 5% $CO_2$ (22). Untreated HUVECS served as the control group. RNA was extracted from the treated cells for subsequent reverse transcription-quantitative (RT-q) PCR.

*Reverse transcription-quantitative (RT-q) PCR.* According to the manufacturer's protocol, RNAiso (Takara Bio, Inc.) was used to extract total RNA from cells, and NanoDrop2000 was used to measure RNA concentration and purity. PrimeScript™ RT Master Mix (Takara Bio, Inc.) was used for reverse transcription according to the manufacturer's instructions. The following protocol was used: 37˚C for 15 min (reverse transcription reaction) and 85˚C for 5 sec (reverse transcriptase inactivation reaction). The cDNA obtained by reverse transcription was amplified on StepOne Plus (Thermo Fisher Scientific Inc.) using the TB Green® Premix Ex Taq™ kit (Takara Bio, Inc.) according to the manufacturer's protocol. The thermocycling conditions used were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and finally annealing and extension at 60˚C for 30 sec. The relative expression level was calculated using the $2^{-\Delta\Delta Cq}$ method (23). The following primers were used: GAPDH forward, 5'-GGA GCCAAAAGGGTCAT-3' and reverse, 5'-GAGTCCTTC CACGATACCAA-3'; X-linked inhibitor of apoptosis protein (XIAP) forward, 5'-GTGACTAGATGTCCACAAGG-3' and reverse, 5'-GTTGAGGAGTGTCTGGTAAG-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCT TCACGAATTTGCGT-3'; and miR-616-3p forward, 5'-ACA CTCCAGCTGGGAGTCATTGGAGGGTTT-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'. GAPDH was used as the internal control for XIAP and U6 was used as the internal control for miR-616-3p.

*Cell counting kit-8.* HUVECs were seeded into 96-well plates at a density of 4,000 cells per well and the culture plate was placed in the incubator for 24 h at 37˚C. Cell counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc.) solution (10 µl) was added to each well and the culture plate was placed in the incubator for 1 h at 37˚C. Absorbance was measured at 450 nm with a microplate reader (iMark™ Microplate Absorbance Reader; Bio-Rad Laboratories Inc.), and the measured optical density (OD) value was used as an indicator of cell viability.

*Transfection.* miR-616-3p mimic, miR-616-3p mimic non-targeting control, XIAP overexpression plasmid and non-targeting control (empty vector) were designed and synthesized by Shanghai Genepharma, Co. Ltd. miR-616-3p mimic (100 nM), miR-616-3p mimic non-targeting control (100 nM), XIAP overexpression plasmid (4 µg) and non-targeting control (4 µg) were transfected into HUVECs with Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific Inc.) at 37˚C for 48 h. Untransfected cells were used as negative control (NC). RNA and total protein were extracted for subsequent experiments 48 h after transfection. miR-616-3p mimic, 5'-AGUCAUUGGAGGGUUUGAGCA G-3'; and miR-616-3p mimic non-targeting control, 5'-ACU ACUGAGUGACAGUAGA-3'.

*Flow cytometry analysis.* HUVECs ($1x10^6$/well) from negative control, miR-616-3p and miR-616-3p mimic control were collected and washed with pre-cooled PBS and centrifuged at 725 x g for 10 min at 4˚C. The supernatant was discarded and the cells were resuspended in 200 µl binding buffer. Subsequently, 10 µl of Annexin V-FITC and 10 µl propidium iodide (PI) was added (Dead Cell Apoptosis Kit with Annexin V FITC and PI; Thermo Fisher Scientific Inc.) and mixed for 15 min at room temperature in the dark. Finally, 300 µl of binding buffer was added and flow cytometry analysis was performed within 1 h on the FACSCalibur Flow Cytometry System (BD Biosciences), and the results were analyzed using FlowJo v.8.0 software (Tree Star, Inc.). Both early and late apoptosis were assessed.

*TUNEL staining.* HUVECs were seeded into 48-well plates at a density of $10^4$ cells per well. First, the cells were washed with saline. Subsequently, the cells were fixed in PBS with

4% neutral formaldehyde at room temperature for 15 min. Then, the cells were washed once with PBS. After treatment with Enhanced Immunostaining Permeabilization Solution (cat. no. P0097; Beyotime Institute of Biotechnology) for 5 min at room temperature, the cells were washed twice with PBS. Then, the slides were incubated with TUNEL reaction mixture (One Step TUNEL Apoptosis Assay kit; cat. no. C1090; Beyotime Institute of Biotechnology) for 60 min at 37˚C. The cells were then immediately observed under a fluorescence microscope to observe the red fluorescence (magnification, x400).

*Western blotting*. HUVECs (1x10$^6$/well) were washed once with ice cold PBS and lysed with RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) on ice for 30 min. The homogenate was collected and centrifuged at 14,000 x g for 10 min at 4˚C. The supernatant was the total protein and was used to determine the protein concentration with the bicinchoninic acid (BCA) method. Next, 40 µg of the extracted protein was added to each well for 10% SDS-PAGE electrophoresis and the protein was transferred to a PVDF membrane. The PVDF membrane was blocked with 5% skimmed milk containing TBS-0.1% Tween-20 (TBS-T) at room temperature for 1 h and incubated with diluted primary antibodies (all Abcam) against β-actin (1:1,000; cat. no. ab8227), XIAP (1:1,000; cat. no. ab229050), cleaved caspase-3 (1:1,000; cat. no. ab32042) and total caspase-3 (1:1,000; cat. no. ab32150) at 4˚C overnight. The PVDF membrane was then washed with TBST and incubated with the corresponding diluted secondary antibodies (1:5,000; cat. no. ab205718; Abcam) at room temperature for 1 h. BeyoECL Moon (Beyotime Institute of Biotechnology) was added to the PVDF membrane to detect the chemiluminescence intensity. Image J software v.2.1.4.7 (National Institutes of Health) was used to analyze band intensity. β-actin was used as the loading control.

*Dual-luciferase reporter assay*. Based on the StarBase 3.0 (http://starbase.sysu.edu.cn/) prediction, miR-616-3p was found to have a potential binding site for the 3'UTR of XIAP mRNA. Luciferase reporter plasmids containing the wild-type (wt) or mutant (mut) 3'untranslated region (UTR) sequence of XIAP. pmirGLO-XIAP-wt and pmirGLO-XIAP-mut plasmids were constructed by Shanghai Gene Pharma Co. Ltd. HUVECs were seeded in 24-well plates at a density of 2x10$^5$ cells/well. miR-616-3p mimic (5'-AGUCAUUGGAGG GUUUGAGCAG-3') and miR-616-3p non-targeting control (5'-ACUACUGAGUGACAGUAGA-3') were synthesized by Shanghai Gene Pharma Co. Ltd. According to the manufacturer's instructions, Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific Inc.) was used to transfect miR-616-3p mimic, miR-616-3p non-targeting control, pmirGLO-XIAP-wt and pmirGLO-XIAP-mut into cells. After 48 h of transfection, the medium was removed and fluorescence intensity was detected using the Dual Luciferase Reporter Gene Assay kit (Beyotime Institute of Biotechnology). *Renilla* luciferase activity was used as the normalization control.

*Statistical analysis*. All experiments were repeated 3 times. All results are expressed as mean ± SD. Statistical analysis was performed using SPSS 19.0 (IBM Corp.). Paired Student's t-test was used for comparison between 2 groups. ANOVA followed by a post hoc Tukey's test was used for comparison between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

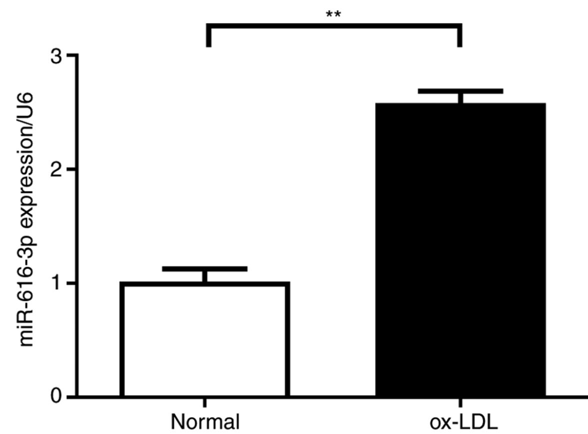


Figure 1. Ox-LDL treatment increases the expression of miR-616-3p in HUVECs compared with normal HUVECs when analyzed with RT-qPCR. **P<0.001. Ox-LDL, oxidized low-density lipoprotein; miR, microRNA; normal, untreated HUVECs.

## Results

*ox-LDL treatment increases miR-616-3p levels in HUVECs*. RT-qPCR analysis of HUVECs treated with ox-LDL revealed that compared with normal HUVECs, ox-LDL treatment resulted in an increase in the expression of miR-616-3p (Fig. 1).

*miR-616-3p inhibits viability and promotes apoptosis of HUVECs*. Firstly, HUVECs were transfected with miR-616-3p mimic and the corresponding non-targeting control. The results confirmed that compared with miR-616-3p mimic non-targeting control, miR-616-3p mimic increased miR-616-3p expression (Fig. 2A). Subsequently, the viability of HUVECs was assessed using the CCK-8 assay. The results demonstrated that compared with miR-616-3p mimic non-targeting control, miR-616-3p mimic significantly inhibited the viability of HUVECs (Fig. 2B). Flow cytometry analysis and TUNEL staining were used to analyze apoptosis of HUVECs. Flow cytometry analysis demonstrated that compared with miR-616-3p mimic control, miR-616-3p mimic significantly increased cell apoptosis (Fig. 2C). The results of the TUNEL staining experiment were consistent with the results of flow cytometry analysis (Fig. 2D).

*miR-616-3p directly inhibits XIAP expression*. Next, the present study explored the mechanism via which miR-616-3p inhibits viability and promotes apoptosis in HUVECs. Based on the StarBase 3.0 prediction, miR-616-3p was found to have a potential binding site for the 3'UTR of XIAP mRNA (Fig. 3A). Subsequently, whether ox-LDL treatment would cause changes in XIAP expression was assessed. Using RT-qPCR, it was demonstrated that contrary to its effect on miR-616-3p, ox-LDL treatment inhibited the expression of XIAP (Fig. 3B). Next, the effect of miR-616-3p on

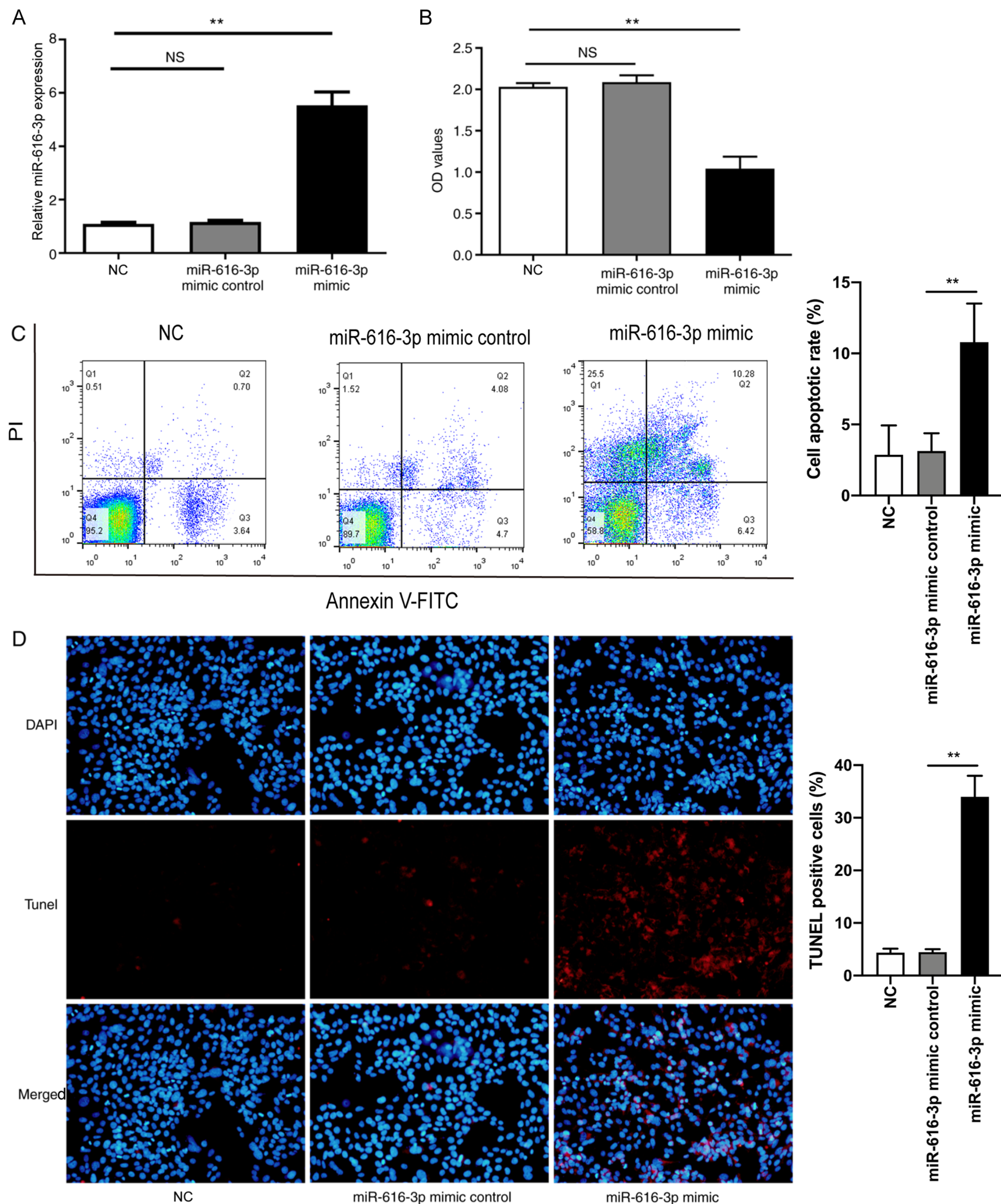


Figure 2. Effect of miR-616-3p on the viability and apoptosis of HUVECs. (A) Transfection of miR-616-3p mimic and miR-616-3p mimic control. Compared with miR-616-3p mimic control, miR-616-3p mimic increased the expression of miR-616-3p in HUVECs and (B) inhibited the viability of HUVECs. (C) Flow cytometry analysis showed that the miR-616-3p mimic increased the apoptosis rate of HUVECs cells. (D) TUNEL staining showed that the miR-616-3p mimic promoted apoptosis of HUVECs (magnification, x400). $^{**}$P<0.001. Ox-LDL, oxidized low-density lipoprotein; miR, microRNA; NC, untreated HUVECs; PI, propidium iodide; OD, optical density.

XIAP in HUVECs was assessed using western blotting. The results demonstrated that compared with miR-616-3p mimic non-targeting control, miR-616-3p mimic inhibited XIAP protein expression (Fig. 3C). Finally, the direct interaction between miR-616-3p and XIAP was demonstrated through dual-luciferase experiments. The results revealed that compared with co-transfection of miR-616-3p mimic and pmirGLO-XIAP-mut, the fluorescence intensity of cells treated with pmirGLO-XIAP-wt and the miR-616-3p mimic decreased significantly (Fig. 3D). This indicated that miR-616-3p directly acts on XIAP mRNA to inhibit XIAP gene expression (Fig. 3D).

*Inhibition of miR-616-3p expression inhibits the expression of cleaved caspase-3 protein in HUVECs.* Cleaved caspase-3 is an apoptosis-related protein (24). Since mR-616-3p was found to promote apoptosis in HUVECs, western blotting analysis was used to determine whether miR-616-3p affects the expression of cleaved caspase-3 protein. Compared with non-targeting control, XIAP

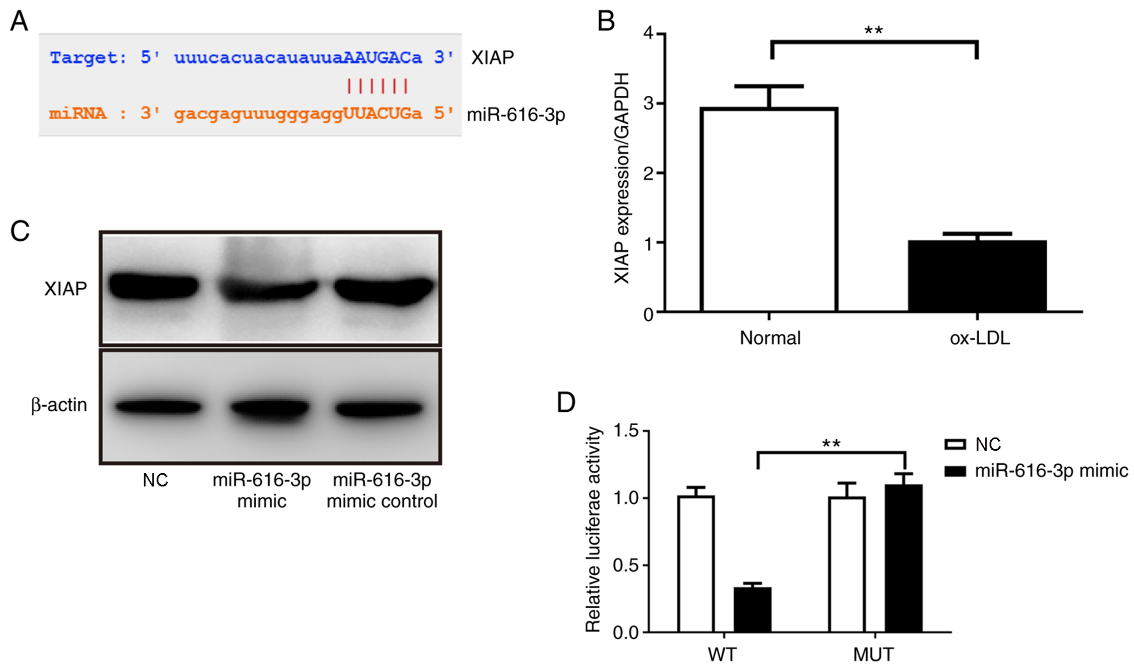


Figure 3. Effect of miR-616-3p on XIAP expression. (A) According to StarBase v.3.0 (http://starbase.sysu.edu.cn/), the binding site on miR-616-3p for the 3'UTR of XIAP. (B) XIAP expression of HUEVCs after Ox-LDL treatment was lower compared with that of the control group. (C) Compared with miR-616-3p mimic control, miR-616-3p mimic inhibited the expression of XIAP of HUVECs cells. (D) The fluorescence intensity of pmirGLO-XIAP-wt co-cultured with the miR-616-3p mimic was significantly reduced. $^{**}$P<0.001. Ox-LDL, oxidized low-density lipoprotein; miR, microRNA; NC, untreated HUVECs; WT, wild-type; mut-mutant; XIAP, X-linked inhibitor of apoptosis protein.

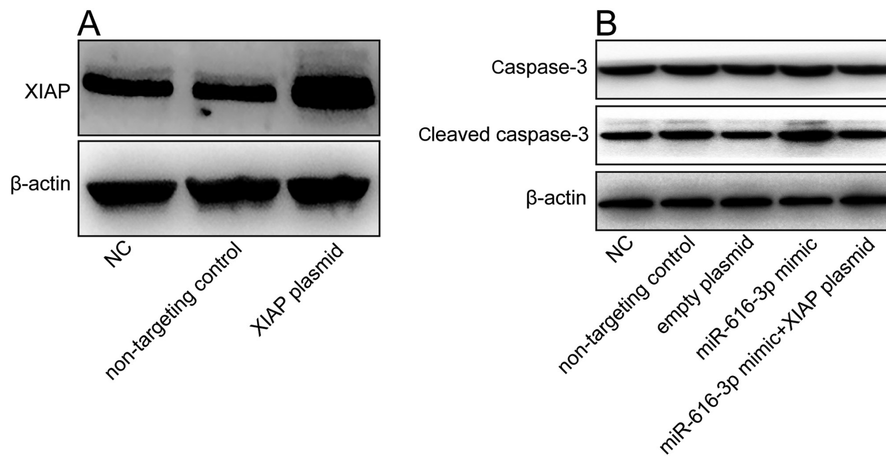


Figure 4. Effect on miR-616-3p on the expression of cleaved caspase-3 and the counter effect of XIAP. (A) XIAP protein expression levels were detected by western blotting and XIAP overexpression plasmid significantly increased XIAP expression in HUVECs. (B) Western blotting was used to detect XIAP protein expression levels, miR-616-3p mimic promoted the expression of cleaved caspase-3 protein in HUVECs and this effect was counteracted by the XIAP overexpression plasmid. Ox-LDL, oxidized low-density lipoprotein; miR, microRNA; NC, untreated HUVECs; XIAP, X-linked inhibitor of apoptosis protein..

overexpression plasmid significantly increase the expression of XIAP (Fig. 4A). Co-transfection of miR-616-3p mimic and XIAP overexpression plasmid inhibited cleaved caspase-3 protein expression in HUVECs (Fig. 4B). This indicated that the miR-616-3p mimic promoted apoptosis of HUVECs by inhibiting XIAP.

*XIAP overexpression plasmid counteracts the effect of the miR-616-3p mimic on the viability and apoptosis of HUVECs*. Using the CCK-8 assay it was demonstrated that miR-616-3p mimic inhibited HUVEC viability and this effect was partially counteracted by the XIAP overexpression plasmid (Fig. 5A). Similarly, in the flow cytometric analysis and TUNEL staining experiments, it was found that the miR-616-3p mimic promoted HUVEC apoptosis and the XIAP overexpression plasmid counteracted this effect (Fig. 5B and C).

## Discussion

The present study investigated the role of miR-616-3p in endothelial cell apoptosis and the potential mechanisms that may be involved in the context of atherosclerosis. The results of flow cytometry and TUNEL staining in the present study

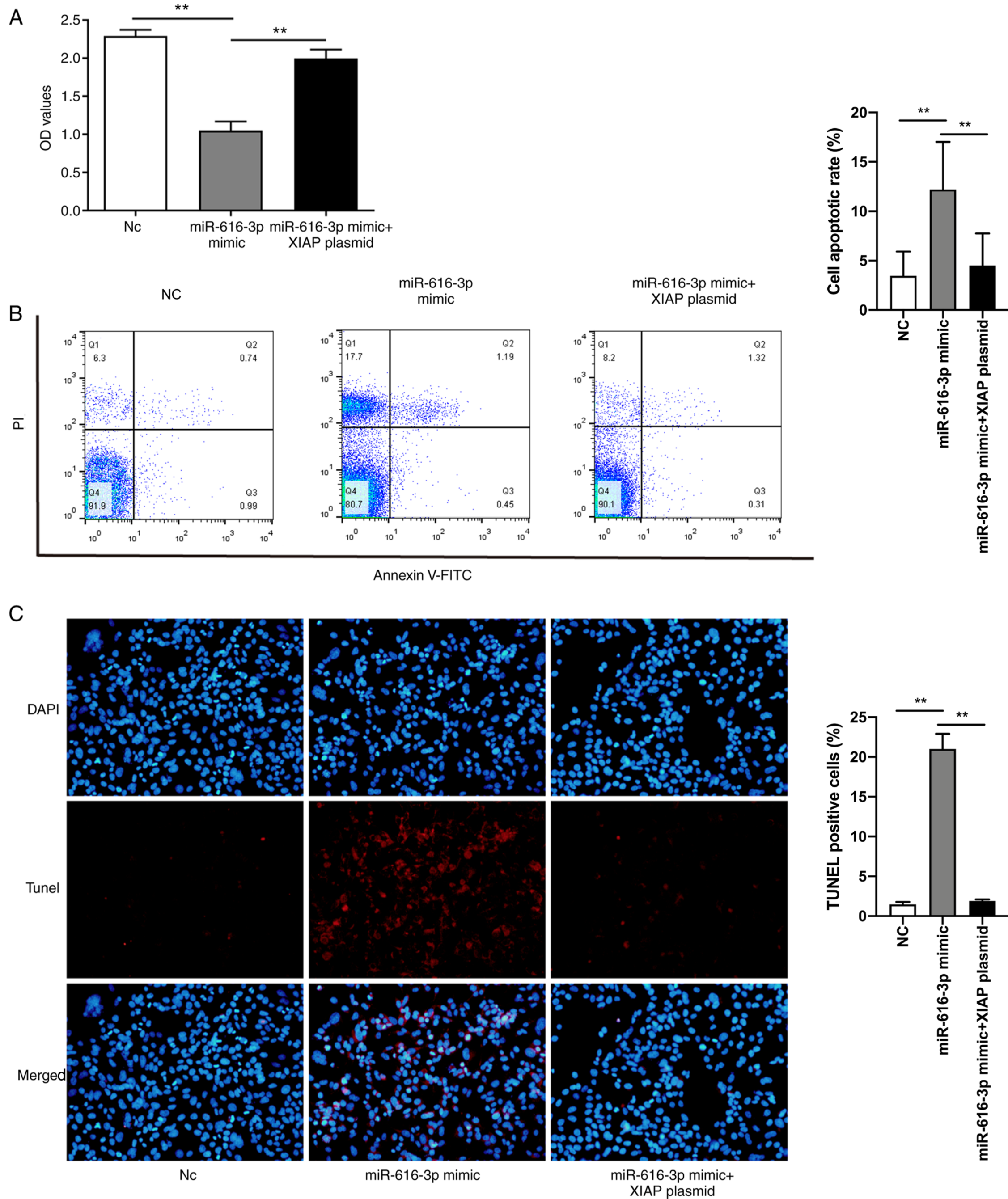


Figure 5. Counter effect of XIAP on the apoptosis-promoting and viability-inhibiting effects of miR-616-3p on HUVECs. (A) miR-616-3p mimic inhibited the viability of HUVECs and this effect was counteracted by the XIAP overexpression plasmid. (B) Flow cytometry results demonstrated that the miR-616-3p mimic promoted HUVEC apoptosis, but the XIAP overexpression plasmid counteracted this effect. (C) TUNEL staining results demonstrated that the miR-616-3p mimic increased the apoptosis rate of HUVECs, and this effect was also counteracted by the XIAP overexpression plasmid (magnification, x400). **P<0.001. Ox-LDL, oxidized low-density lipoprotein; miR, microRNA; NC, untreated HUVECs; PI, propidium iodide; OD, optical density; XIAP, X-linked inhibitor of apoptosis protein.

demonstrated that miR-616-3p significantly promoted HUVEC apoptosis. This finding of the present study confirmed the role of miR-616-3p in atherosclerosis via promotion of apoptosis in endothelial cells. Using StarBase3.0, the present study predicted that miR-616-3p may bind to the XIAP mRNA to induce its effects on HUVECs. Shin *et al* (25) demonstrated that miR-513a-5p mediates tumor necrosis-α and lipopolysaccharide induced apoptosis via downregulation of XIAP in HUVECs. Another study by Li *et al* (26) demonstrated that miR-122 promotes endothelial cell apoptosis by targeting XIAP.

Hence, the present study examined whether miR-616-3p promoted apoptosis of endothelial cells by directly acting on the 3'UTR of XIAP and inhibiting the expression of XIAP. In the present study, ox-LDL treatment resulted in an increase in miR-616-3p expression and decrease in XIAP expression in HUVECs. In addition, dual-luciferase experiments performed in the present study demonstrated that miR-616-3p mimic can directly target the XIAP 3'UTR. Flow cytometry and TUNEL staining experiments performed in the present study also confirmed that miR-616-3p mimic can promote apoptosis

of HUVECs and this effect can be partially reversed by the XIAP overexpression plasmid.

Caspase-3 is the most important terminal cleavage enzyme in the process of apoptosis and cleaved-caspase-3 is the activated form of caspase-3 (27). Through western blotting changes in the expression of the apoptosis-related protein cleaved caspase-3 were found in the present study. In the present study, compared with miR-616-3p non-targeting control, miR-616-3p mimic increased the expression of cleaved caspase-3 protein and this effect was partially reversed by the XIAP overexpression plasmid.

The present study had several limitations. Firstly, the specificity of miR-166-3p and the causal relationship between miR-166-3p and endothelial cell apoptosis need to be further verified. Secondly, only *in vitro* cell experiments were conducted in the present study and future *in vivo* experiments are needed to verify the findings of the present study.

In summary, the present study found that miR-616-3p can directly act on the 3'UTR of XIAP to promote apoptosis of HUVECs. The present study provides a new basis for the pathogenesis of atherosclerosis and indicates that miR-616-3p may have potential as a treatment target in the future.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

XZ designed the experiments. HC, XL, YW, XWu, XWen and YL performed the experiments. HC collected and analyzed the data. All authors confirmed the authenticity of the raw data. HC and XZ wrote the manuscript. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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