European Review for Medical and Pharmacological Sciences 2019; 23: 4491-4497

Effect of lncRNA H19 on the apoptosis of vascular endothelial cells in arteriosclerosis obliterans via the NF-κB pathway

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Abstract. – OBJECTIVE: To explore the effect of long non-coding ribonucleic acid (lncRNA) H19 on the apoptosis of vascular endothelial cells in arteriosclerosis obliterans (ASO) via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway.

PATIENTS AND METHODS: Human umbilical vein endothelial cells (HUVECs) were cultured, and lncRNA H19 was inhibited by Si-H9 and overexpressed by H19-OE. Then, the apoptosis rate was detected by flow cytometry, the target of lncRNA H19 was detected by dual luciferase reporter gene assay, and changes in the protein level were determined via Western blotting (WB).

RESULTS: LncRNA H19 exhibited high expression in serum of patients with ASO, and compared with that in congeneric normal mice, the expression of lncRNA H19 in ASO mice rose. Besides, the proliferation ability of cells transfected with H19-OE was markedly strengthened, and H19-OE treatment could down-regulate the expression level of the apoptin, active cysteinyl aspartate-specific proteinase-3 (Caspase-3). In addition, lncRNA H19 bound to micro ribonucleic acid (miR)-19a in a targeted way. After lncRNA H19 was overexpressed, the expression of the NF-κB pathway key factors, p38 and p65, were notably increased, and the nuclear translocation of p65 was significantly enhanced after transfection with miR-19a.

CONCLUSIONS: LncRNA H19 promotes the proliferation of vascular endothelial cells in ASO and inhibits the apoptosis of them via the NF-κB pathway.

Key Words

LncRNA H19, Vascular endothelial cells, Arteriosclerosis obliterans, NF-κB pathway.

Introduction

Despite the magnificent progress in basic and clinical research of atherosclerosis in recent years, it remains one of the most common cardiovascular diseases, with the highest mortality rate worldwide[1]. As a local manifestation of systemic atherosclerosis in limbs, arteriosclerosis obliterans (ASO) refers to a degenerative and proliferative change in the systemic arterial intima and its middle layer, which causes the hardened and shrinking vessel wall and elasticity loss, resulting in secondary thrombosis and progressive reduction or interruption of the distal blood flow. Besides, ASO is a multi-step disease induced by varying risk factors, including accumulation of macrophages, pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), endothelial dysfunction and vascular smooth muscle cells (VSMCs)[2]. ASO is very likely to start with endothelial dysfunction, which expresses adhesion molecules, attracts different monocytes, and leads to inflammatory responses[3]. Varying dysfunction of endothelial cells and VSMCs are pivotal cellular events resulting in arteriosclerosis, including abnormal proliferation, migration, apoptosis, and abnormally expressed adhesion molecule proteins. The functions of endothelial cells and VSMCs, therefore, are crucial for the treatment of ASO.

Long non-coding ribonucleic acids (lncRNAs) participate in many biological and pathological processes, such as carcinogenesis and chronic diseases[4]. Their epigenetic regulation on the gene expression at the transcriptional level involves multiple signal pathways[5]. Scholars have reported that lncRNAs exert a crucial effect on regulating atherosclerosis. Wu et al[6] reported that lncRNA p21 can inhibit the proliferation of VSMCs and induce the apoptosis of them in apolipoprotein E (ApoE)$^{-/-}$ mice by enhancing the transcription activity of p53. Ballantyne et al[7] found that lncRNA smooth muscle-induced lncRNA (SMILR) can regulate the proliferation of VSMCs and be highly expressed in unstable atherosclerotic plaques in the human. The above results indicate that the regulation on SMILR may be a new therapeutic

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regimen to prevent vascular diseases. Bao et al[8] detected differentially expressed lncRNAs in ApoE$^{-/-}$ mice treated with a high-fat diet using a microarray, and they identified 354 differentially expressed lncRNAs (>2-fold changes), but the function and mechanism of these lncRNAs remain unclear. These studies show that lncRNAs are very important in regulating atherosclerosis. To determine the specific function and mechanism of lncRNAs, further research is needed.

LncRNA H19 is transcribed by H19/IGF2 gene located on human chromosome 11p15.5[9]. Recent studies have revealed that H19 is closely associated with various tumors through different signaling pathways. It was found by Gao et al[10] that H19 is related to the risk and severity of ASO in the Chinese population, indicating that H19 may be a potential target for the treatment of atherosclerosis. However, the role and possible mechanism of H19 in ASO are still unclear.

Therefore, this study aims to detect the expression of lncRNA H19 in ASO and its molecular mechanism.

Patients and Methods

Clinical Specimens

Serum samples of 62 patients with atherosclerosis treated in the Department of Cardiology of our hospital from January 2018 to December 2018 and those of 35 healthy volunteers were collected. All patients and healthy volunteers signed the informed consent. The experimental process was approved by the Ethics Review Committee of our hospital.

Cell Culture and Experimental Animals

Human umbilical vein endothelial cells (HUVECs) were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C. The culture medium was replaced every other day, and the cells passaged when the cell fusion degree reached 80-90%.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Then, the total RNA was synthesized into the complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT kit (TaKaRa, Otsu, Shiga, Japan). Finally, the following specific primers were applied for the PCR (Table I).

Detection of Apoptosis Via Flow Cytometry

The cell culture media were collected separately into the centrifuge tube after different treatments. The cells were washed with phosphate-buffered saline (PBS) twice, also collected, respectively, and moderately digested with trypsin. After that, the old culture solution collected before was used to terminate the cell digestion in each group. After blowing, all the cells were collected into the centrifuge tube for centrifugation at 1000 rpm for 5 min, and the supernatant was discarded. Then, the cells were resuspended with 1 mL pre-cooled PBS solution in each group and centrifuged at 1000 rpm for 5 min, and the supernatant was discarded, 50 μL of which was reserved. Subsequently, all the cells were transferred to a 1.5 mL EP tube and again washed with PBS once, and the supernatant was discarded. Then, the cells were treated with rJHP0290 (100 ng/mL) and/or CPT (10 μM) (Sigma-Aldrich, St. Louis, MO, USA). According to the manufacturer's instructions, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V antibodies and Propidium Iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA). In short, 1×10^5 cells in 100 μL annexin binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM

Table I. Primer sequences.

Gene	Forward primer sequence	Reverse primer sequence
P65	5'-CCCACAACGAAATCATGACAAG-3'	5'-GAGGTACGCCAGGAATTGTTG-3'
MiR-19a-3p	5'-CTGGAGAATAACAGAGGGATGC-3'	5'-CCTGGCTCCTCACTTGGC-3'
MiR-19b-3p	5'-GATCCCTGATGCTGTCTTC-3'	5'-AGGGTCCTTCTGGTTCTTG-3'
β-actin	5'-AAGTACTCCGTGTGGATCGG-3'	5'-ATGCTATCACCTCCCCTGTG-3'

$CaCl_2$) were mixed with 1 µL FITC-conjugated annexin V antibody. The mixture was then incubated in the dark at room temperature for 15 min, and PI (2.5 µL) was added to the cell suspension immediately prior to analyze them. Finally, the relative number of annexin V-positive and/or PI-positive cells was determined using flow cytometry, and FlowJo software was applied for data analysis.

Western Blotting (WB)

The protein was extracted with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. After the protein was successfully transferred, the membrane was sealed with 5% skim milk at 37°C for 2 h and incubated with primary antibodies at 4°C overnight. The membrane was then incubated with secondary antibodies conjugated with horseradish peroxidase at 37°C for 1 h. According to the manufacturer's instructions, the bands were observed with an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA, USA).

Determination of Cell Viability

The cells were inoculated into a 96-well plate at a concentration of $4x10^3$ cells/well. When the fusion reached about 70%, the cells were treated with apigenin (APG) (10 µM), miR-101 mimics (50 nM) and APG combined with miR-101 mimics and then incubated in fresh medium containing doxorubicin (ADM) (0.5-8 µM) for 48 h. Subsequently, 20 µL 3-(4,5)-dimethylthiazol (-z-yl)-3,5-diphenyltetrazolium bromide (MTT) solution (2 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added to the wells for incubation for 4 h, and 200 µL dimethyl sulfoxide was added to each well to dissolve the formed methyl crystals. Ultimately, the optical density was measured at 570 nm using a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc, Chicago, IL, USA) was adopted for statistical analysis. All the results were expressed as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). Then, SPSS 17.0 and GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) were used. $p<0.05$ represented that the difference was statistically significant.

Results

LncRNA H19 was Highly Expressed in Patients with ASO

To detect the role of H19 in ASO, the expression of lncRNA H19 in the serum of patients was first detected. It was found that, compared with that in healthy volunteers, lncRNA H19 was highly expressed in the serum of patients ($p<0.001$) (Figure 1A). Besides, the expression of lncRNA H19 in atherosclerotic plaques of $ApoE^{-/-}$ mice was also detected, and the results revealed that the expression of lncRNA H19 in ASO mice was increased compared with that in congeneric normal mice ($p<0.001$) (Figure 1B). The above results indicate that lncRNA H19 may be a potential risk factor for ASO.

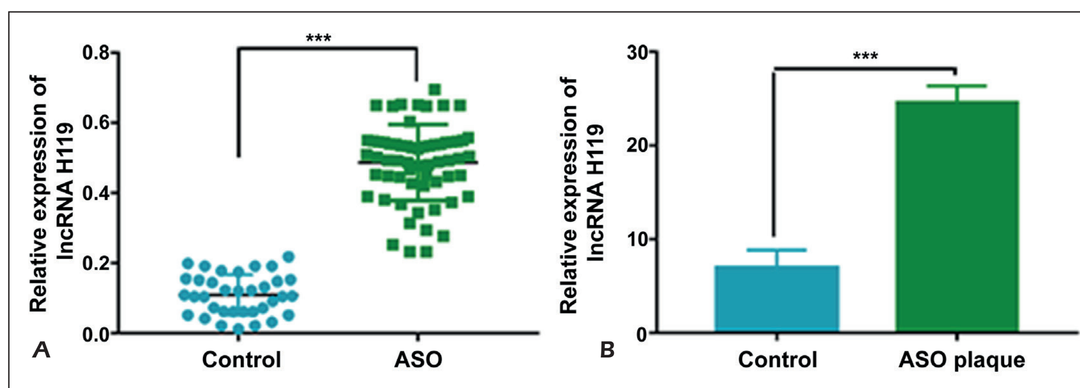

Figure 1. A, Quantitative RT-PCR (qRT-PCR) is conducted to detect the expression of lncRNA H119 in serum of 62 patients with ASO and 35 healthy volunteers. **B**, Expression of lncRNA H19 in $ApoE^{-/-}$ mice with ASO is also detected *via* qRT-PCR.

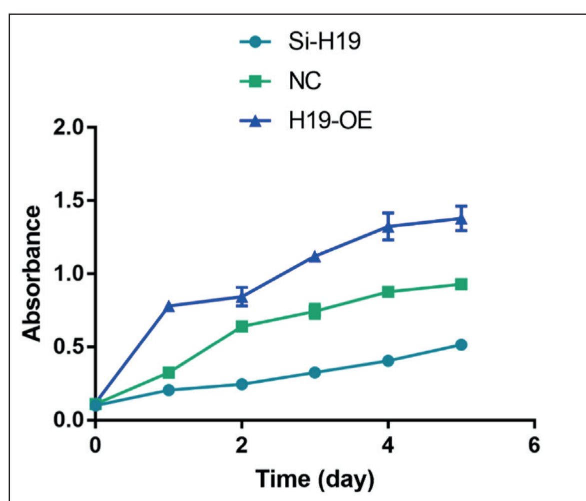

Figure 2. Cell proliferation ability detection. The proliferation ability of cells transfected with Si-H9 is markedly weakened, while that of cells transfected with H19-OE is remarkably strengthened ($p<0.01$).

LncRNA H19 Promoted the Proliferation of HUVECs

The proliferation ability of HUVECs was detected after they were transfected with Si-H9 and H19-OE, respectively. The proliferation ability of HUVECs transfected with Si-H9 was markedly weakened, while that of HUVECs transfected with H19-OE was notably enhanced ($p<0.01$). The above results suggest that lncRNA H19 can promote cell proliferation ($p<0.01$) (Figure 2).

LncRNA H19 Inhibited Cell Apoptosis

After HUVECs were transfected with Si-H19, the apoptosis rates of them were detected *via* flow cytometry. The apoptosis rates of Si-H19 group and normal control (NC) group were (26.45±4.62)% and (13.57±2.35)%, respectively, with a statistically significant difference ($p<0.05$) (Figure 3A).

WB was adopted to detect the expression of active aspartate-specific proteinase-3 (Caspase-3) in each group of HUVECs after they were transfected with H19-OE. It was found that compared with NC, H19-OE treatment could reduce the expression level of the apoptin active Caspase-3 ($p<0.05$) (Figure 3B).

LncRNA H19 Bound to MiR-19a in a Targeted Way

The target of lncRNA H19 was predicted using bioinformatics. According to the results, lncRNA H19 contained the complementary sites of the 3'untranslated regions (3'UTRs) of miR-19a and miR-19b (Figure 4A). After lncRNA H19 was overexpressed, only the expression level of miR-19a was markedly decreased ($p<0.05$) (Figure 4B), indicating that lncRNA H19 binds to miR-19a in a targeted way.

LncRNA H19 Regulated Vascular Endothelial Cells in ASO Through the Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB) Pathway

To further clarify the mechanism of lncRNA H19 regulating vascular endothelial cells in ASO, bioinformatics analysis manifested that, after the overexpression of lncRNA H19, the NF-κB pathway was activated. Further cytological experiments manifested that, after lncRNA H19 was overexpressed, the expressions of the NF-κB pathway key factors, p38 and p65, were evidently increased (Figure 5).

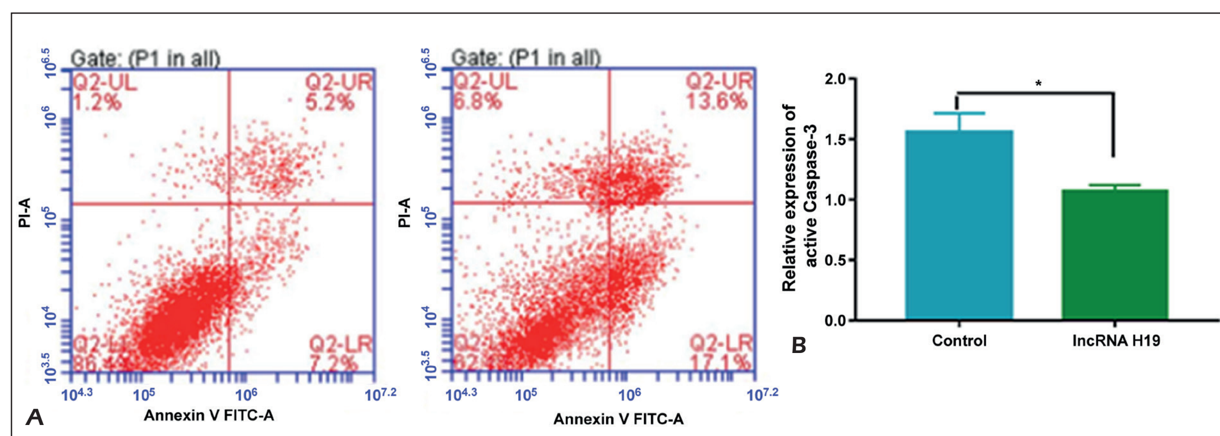

Figure 3. A, Apoptosis rate detected by flow cytometry. The apoptosis rates of Si-H19 group and NC group are (26.45±4.62)% and (13.57 ±2.35)%, respectively. **B**, Expression of active Caspase-3 in each group of cells detected via WB: H19-OE treatment can down-regulate the protein expression level of the apoptin active Caspase-3 ($p<0.05$).

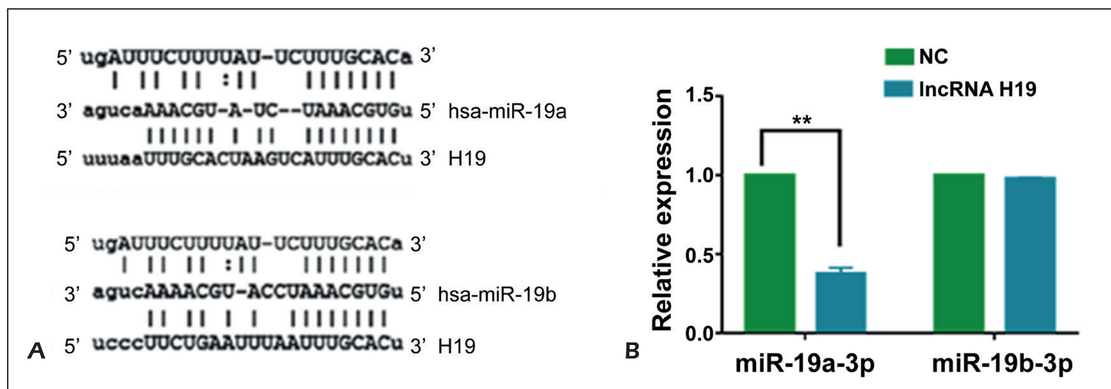

Figure 4. *A*, LncRNA H19 contains the complementary sites of 3'UTRs of miR-19a and miR-19b. **B**, After lncRNA H19 is overexpressed, the expression levels of miR-19a and miR-19b are detected.

The nuclear localization of NF-κB subunit p65 was analyzed. Immunofluorescence and Western blotting analysis revealed that the nuclear translocation of p65 was significantly enhanced after transfection with miR-19a ($p<0.05$) (Figure 6).

Discussion

We found that lncRNA H19 was highly expressed in the serum of patients with ASO and ApoE$^{-/-}$ mice with ASO. The high expression of lncRNA H19 in HUVECs can lead to an increased proliferation and decreased apoptosis.

According to recent studies[11,12], non-coding RNAs play a crucial role in regulating atherosclerosis. It was reported by Huang et al[13] that lncRNA HOXC-AS1 is decreased in atherosclerosis and can inhibit oxidized low-density lipoprotein (ox-LDL)-induced cholesterol accumulation in THP-1 macrophages through promoting the HOXC6 expression. The above findings indicate that lncRNA HOXC-AS1 can be used to treat atherosclerosis. Besides, Shan et al[14] also found that lncRNA RNCR3 is highly expressed in ASO in mice and the human. The down-regulated lncRNA RNCR3 will lead to reduced proliferation and expression of miRNAs in ECs and VSMCs, which indicates that lncRNA RNCR3 may be a potential target for the treatment of ASO. Furthermore, Bao et al[7] found that 354 differentially expressed lncRNAs are closely associated with the pathological changes of ApoE$^{-/-}$ mice fed with a high-fat diet, which may play a role in the inflammation and metabolism of ASO. The role of lncRNA H19 in ASO was the focus of this study, and it was found that lncRNA H19 was highly expressed in ApoE$^{-/-}$ mice with atherosclerotic plaques and serum of patients with atherosclerotic plaques, indicating that lncRNA H19 may be applied as a biomarker for ASO diagnosis.

Endothelial cells, VSMCs and macrophages are the main cells contributing to the formation of arteriosclerosis and pathological changes[15,16]. The formation of lipid carrier foam cells is induced by macrophage ox-LDL, which can produce a variety of pro-inflammatory cytokines, such as matrix metalloproteinases (MMPs), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α). Under normal circumstances, the proliferation rate of VSMCs is low. In response to ox-LDL and activated macrophages, VSM can migrate to the arterial intima and proliferate and produce fibrous tissues, thus forming plaques. What's more, these activated VSMCs can also produce pro-inflammatory cytokines

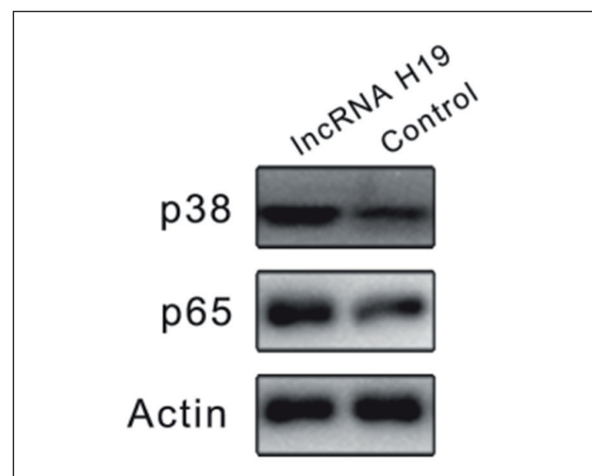

Figure 5. After the overexpression of lncRNA H19, the expressions of the NF-κB pathway key factors, p38 and p65, are significantly increased ($p<0.01$).

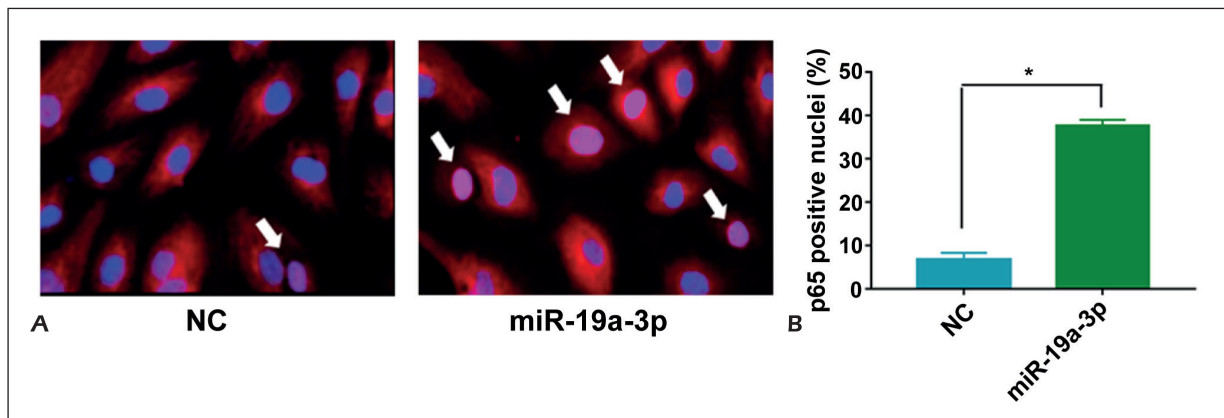

Figure 6. A, Nuclear localization of NF-κB in cells transfected with NC or miR-19a: p65 staining (red). **B**, Quantified percentage of p65 positive nuclei (n=3 in each group) ($p<0.05$).

and phagocytize lipoprotein[13]. It was found in this study that the overexpression of lncRNA H19 in vascular endothelial cells can lead to increased proliferation and decreased apoptosis. Li et al[17] reported that lncRNA H19/miR-675 can reduce inflammation and apoptosis of myocardial cells in diabetic cardiomyopathy. The results of this study are consistent with those of previous reports, suggesting that lncRNA H19 may be a key factor in activating vascular endothelial cells.

In addition, it has been observed that mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways are involved in regulating arteriosclerosis. Lee et al[18] reported that lobastin can inhibit the expression of VSM vascular cell adhesion molecule-1 (VCAM-1) in mice through the MAPK and NF-κB signaling pathways, indicating that MAPK and NF-κB may regulate the adhesion ability of VSMCs. Zhao et al[19] revealed that there is a close correlation of heat shock protein 60 (HSP60) with the pathogenesis of arteriosclerosis, and it may stimulate VSMC migration through the MAPK signaling pathway. Yu et al[20] detected that the high-level glucose can stimulate proliferation and migration of VSMCs in mice through the MAPK and NF-κB signaling pathways, and this effect can be inhibited by rutin[21]. The above studies show that the MAPK and NF-κB signaling pathways are vital regulatory factors for the formation and development of arteriosclerosis, and can be used as potential targets for the treatment of ASO. To further investigate the regulatory mechanism of lncRNA H19 in promoting ASO, the expressions of p38 and p65 were observed after the overexpression of lncRNA H19. It was found that p38 and p65 were highly expressed in VSMCs, suggesting that lncRNA H19 may regulate arteriosclerosis through the NF-κB signaling pathway. Furthermore, we found for the first time that lncRNA H19 promoted proliferation and inhibited apoptosis of vascular endothelial cells in ASO through the NF-κB pathway, thus providing a basis for finding potential targets for treatment of ASO.

Conclusions

We found that lncRNA H19 promotes the proliferation of vascular endothelial cells in ASO and inhibits the apoptosis of them *via* the NF-kB pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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