LncRNA H19 promotes atherosclerosis by regulating MAPK and NF-kB signaling pathway

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Abstract. – OBJECTIVE: Atherosclerosis is one of the leading causes of mortality in the world, which is a multistep cardiovascular disease promoted by several of risk factors. However, the detailed mechanism of atherosclerosis remained unclear. LncRNAs have been proved to play an important role in various of biological and pathological processes, but the role of lncRNA in atherosclerosis largely remained unidentified.

PATIENTS AND METHODS: Blood sample were collected from 42 patients with atherosclerosis and 37 healthy volunteers. The expression of lncRNA H19 was detected by the qRT-PCR assay. Proliferation and apoptosis of HUVEC were also detected after lncRNA H19 was overexpressed. The expression of p38 and p65 were also measured by Western blot.

RESULTS: Compared with the normal healthy people, the expression of H19 was higher in patients with atherosclerosis. After lncRNA H19 was overexpressed in HUVEC, the proliferation ability was increased while apoptosis was suppressed. What’s more, p38 and p65 were increased after lncRNA H19 was overexpressed.

CONCLUSIONS: LncRNA H19 was highly expressed in atherosclerosis, which could be used as a potential target for treating atherosclerosis.

Key Words: LncRNA H19, MAPK, NF-kB, Atherosclerosis, Vascular smooth muscle cell.

Introduction

Despite great advance in both basic and clinical research in atherosclerosis, it is still one of the most common cardiovascular diseases at present, which is one of the leading causes of mortality in the world. Atherosclerosis is a multistep disease, which is promoted by various of risk factors, including accumulation of macrophages, production of pro-inflammatory cytokines (such as tumor necrosis factor α and IL-6), dysfunction of endothelial and VSMC (Vascular Smooth Muscle Cell), and so on. Atherosclerosis is likely to start with dysfunction of endothelial, which expresses adhesion molecules, attracting different mononuclear leukocytes, leading to inflammatory reactions. Multiple dysfunctions of endothelial and VSMC are critical cellular events contributing the formation of atherosclerosis, including inappropriate proliferation, migration, apoptosis, and the abnormal expressed adhesion molecular proteins. Thus, the function of endothelial and VSMC are important for the treatment of atherosclerosis.

Long non-coding RNA (lncRNA) has been reported to participate in a lot of biological and pathological processes, such as carcinogenesis and chronic diseases. LncRNA could regulate gene expression epigenetically in transcription and post-transcription level, which is involved in multiple signaling pathways. Recently, several works have reported that lncRNAs were pivotal in the regulation of atherosclerosis. Wu et al. reported that lncRNA p21 could repress cell proliferation and induce apoptosis in the vascular smooth muscle cells in ApoE-/- mice via enhancing p53 transcriptional activity. Ballantyne et al. found that lncRNA SMILR could regulate the proliferation of vascular smooth muscle cells, and it was highly expressed in the human unstable atherosclerotic plaques. Those results indicated that modulation of SMILR might be a novel therapeutic strategy to prevent vascular lesion. Bao et al. detected the differentially expressed lncRNA in ApoE-/- mice treated with high-fat diet using microarray. They identified 354 differentially expressed lncRNAs (>2 fold change), but the function and the mechanism of these lncRNAs remained unclear. These studies indicated that lncRNA was important in regulating atherosclerosis. To identify its detailed function and mechanism, further studies are needed.
LncRNA H19 is transcribed from H19/IGF2 gene, which is located on human chromosome 11p15.513,14. Recent studies have found that H19 was closely related with many kinds of tumors, via different signaling pathways. Gao et al15 found that H19 was associated with the risk and severity of atherosclerosis in a Chinese population, indicating that H19 could be a potential target for treating atherosclerosis. However, the effect and possible mechanism of H19 in atherosclerosis remained unclear.

In this paper, we aimed to detect the expression of lncRNA H19 in atherosclerosis, as well as to recover molecular mechanisms. We first detected the expression of lncRNA H19 in serum of 40 patients with atherosclerosis. After that, H19 was overexpressed in HUVEC and VSMC. Proliferation and apoptosis were measured. The expression of p38 and p65 were also detected after H19 was overexpressed in VSMC.

**Patients and Methods**

**Patients**

42 serum samples from patients with atherosclerosis and 37 serum samples from healthy volunteers were collected at the Department of Cardiology, the First Affiliated Hospital of Chongqing Medical University, from February 2015 to December 2015. All the patients and healthy volunteers were well informed. The procedure of the experiment was approved by the Institutional Review Board of Chongqing Medical University.

**Animals**

The whole procedure was performed according to the National Institutes of Health (NIH) Animal Use Guidelines. 10 ApoE−/− mice were obtained from (Beijing HFK Bioscience Co., Ltd., Beijing, China). Mice received high-fat diet, and were kept in the standard SPF environment, with 12 hours light and 12 hours dark cycle at a stable temperature of 20-25°C. These mice were sacrificed after 16 weeks, and the atherosclerotic plaque and adjacent tissue were kept in the liquid nitrogen for further use.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) and Vascular Smooth Muscle Cells (VSMC) were purchased from the Institute of Biochemistry Cell Biology (Shanghai, China). The cells were culture at 37°C in a humidified incubator with 5% CO2 and 95% air, and maintained in DMEM (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (100 U/mL/100 µg/mL) (Beyotime, Beijing, China).

**qRT-PCR Assay**

Total RNA from cells and clinical samples were obtained using the RNAiso Plus (TAKARA, Otsu, Shiga, Japan) and Trizol LS Reagent (TAKARA, Otsu, Shiga, Japan) separately. Reverse transcription polymerase chain reaction (RT-PCR) was performed using PrimeScript™ RT reagent Kit (TAKARA, Otsu, Shiga, Japan) according to the manufacturer’s protocol. The levels of mRNA expression were quantified by standard Real-time PCR protocol with SYBR Premix Ex Taq (TAKARA, Otsu, Shiga, Japan). GAPDH was used as a reference gene.

**Western-blot Assay**

The HUVECs were grown in the 6-well plate. Total proteins were extracted by RIPA lysis buffer containing PMSF (Beyotime, Beijing, China). Supernatant protein levels were determined by standard BCA assay. An equal amount of protein (50 µg) was loaded into a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA) after electrophoresis. After blocking non-specific binding sites for 1h at room temperature with 5% non-fat milk in TBST (Boster, Wuhan, China), the membranes were individually incubated for overnight with primary antibody at 4°C. Then respective secondary antibody was used to incubate these membranes for 1h at room temperature. The protein bands were revealed by electrochemiluminescence (ECL) method and imaged using a BioSpectrum Gel Imaging System (Bio-Rad, Hercules, CA, USA).

**Apoptosis Assay**

Caspase 3 activity was used as an indicator of apoptosis in this study. Caspase 3 Colorimetric Protease Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to detect caspase 3 activity of target cells. Each experiment was conducted in triplicate.

**CCK8 Assay**

Target cells were seeded into 96-well plate with the density of 2500 cells per well. 10 µl Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) and
100 μl DMEM with 10% FBS were added to each well and cultured for 4 hours at 37°C. The absorbance at 450 nm was measured. Three controls were set in each group and the whole experiment was repeated three times.

Statistical Analysis

All the results were presented as mean ± standard deviation (SD), and analyzed by one-way analysis of variance (ANOVA), followed by post hoc analysis using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA). *p* value < 0.05 was considered as statistically significant.

Results

**LncRNA H19 was Highly Expressed in Patients with Atherosclerosis**

The study has shown that lncRNA H19 may be a potential risk related to the atherosclerosis in the Chinese population, but the mechanism remained unclear. To detect the role of H19 in atherosclerosis, we first detected the expression of lncRNA H19 in serum samples from 42 patients with atherosclerosis, compared with those from 37 healthy volunteers. The expression of lncRNA H19 in atherosclerotic plaque from ApoE-/- mice was also detected by qRT-PCR. *p*<0.001.

**LncRNA H19 Improved the Proliferation and Suppressed Apoptosis of HUME Cells**

To further explore the role of lncRNA H19 in atherosclerosis, we first overexpressed lncRNA H19 in HUVEC and VSMC (Figure 2A). Studies have proved that the dysfunction of endothelial and smooth muscle cells were the key factors in the formation of atherosclerosis, which included abnormal proliferation and apoptosis. CCK8 and apoptosis assays were used to detect the proliferation and apoptosis ability of HUVEC and VSMC after lncRNA H19 was overexpressed (Figure 2B). We found that proliferation ability was increased while the apoptosis ability was decreased in both cell lines (Figure 2 C-D). These results indicated that lncRNA H19 could promote proliferation and inhibit apoptosis of HUVEC and VSMC, which might accelerate the formation of atherosclerosis.

LncRNA H19 could increase the expression of N-cadherin and downregulate E-cadherin migration ability of VSMC and HUVEC is important in the formation of atherosclerosis. To further explore the role of lncRNA H19 in the regulation of migration ability of VSMC and HUVEC, we de-
LncRNA H19 promotes atherosclerosis by promoting the expression of p38 and p65

We found that overexpression of lncRNA H19 could increase the expression of N-cadherin, while the expression of E-cadherin was suppressed (Figure 3 A-B). This result suggested that lncRNA H19 might promote migration ability of VSMC and HUVEC.

**The Expression of p38 and p65 was Unregulated After H19 and Overexpressed**

As we have found that lncRNA H19 could promote atherosclerosis via regulating the proliferation and apoptosis of HUVEC and VSMC, but the molecular mechanism remained unclear. Studies have found that MAPK and NF-kB signaling pathway were involved in regulating the function of EMT markers. We found that overexpression of IncRNA H19 could increase the expression of N-cadherin, while the expression of E-cadherin was suppressed (Figure 3 A-B). This result suggested that lncRNA H19 might promote migration ability of VSMC and HUVEC.

**Figure 2.** LncRNA H19 promotes proliferation and decrease apoptosis in VSMC and HUVEC. (A) LncRNA H19 was overexpressed in VSMC and HUVEC. (B) CCK8 assay was used to detect the proliferation ability of VSMC and HUVEC, after lncRNA H19 was overexpressed. (C) Caspase 3 activity was used to measure apoptosis of VSMC and HUVEC. *p<0.05, ***p<0.001.

**Figure 3.** LncRNA H19 increase the expression of N-cadherin and decrease E-cadherin. (A) The expression of N-cadherin and E-cadherin in VSMC were detected by WB. (B) The expression of N-cadherin and E-cadherin in HUVEC was detected by WB.
J.-X. Pan

According to these studies, we would like to detect whether lncRNA H19 regulated the proliferation and apoptosis via MAPK and NF-κB signaling pathway. We found that overexpressed H19 could lead to the increase of p38 and p65, which were the key factors of MAPK and NF-κB signaling pathway, respectively (Figure 4 A-B), suggesting that lncRNA H19 could activate the MAPK and NF-κB signaling pathway in HUVEC and VSMC.

Discussion

In the present study, we found that lncRNA H19 was highly expressed in the serum of patients with atherosclerosis and atherosclerotic plaque from ApoE-/- mice. Overexpression of lncRNA H19 in HUVEC and VSMC could lead to increase of proliferation and decrease of apoptosis.

Recent studies have shown that non-coding RNA played an important role in the regulation of atherosclerosis. Huang et al. reported that lncRNA HOXC-AS1 was downregulated in atherosclerosis, which could suppress ox-LDL-induced cholesterol accumulation in THP-1 macrophages via promoting HOXC6 expression. This result suggested that lncRNA HOXC-AS1 could be used for treating atherosclerosis. What's more, Shan et al. also found that lncRNA RNCR3 was highly expressed in mouse and human aortic atherosclerosis. The downregulation of lncRNA RNCR3 would lead to the decreased proliferation and migration in ECs and VSMCs, indicating that lncRNA RNCR3 could be a potential target for treating atherosclerosis. Also, Bao et al. found that a total of 354 differentially expressed lncRNAs were closely related to the pathological changes in high-fat diet on ApoE-/- mice, which might play roles in inflammatory and metabolic processes in atherosclerosis. These studies indicated that lncRNAs might be important factors that involved in the formation and development of atherosclerosis, but their function and mechanism in atherosclerosis remained unknown. Gao et al. found that lncRNA H19 was associated with the risk and severity of coronary artery disease, but the mechanism remained unclear. According to this study, we mainly focused on the role of H19 in atherosclerosis. We found that the expression of lncRNA H19 was highly expressed in the serum of patients with atherosclerosis and atherosclerotic plaque from ApoE-/- mice (Figure 1 A-B), suggesting that lncRNA H19 might be used as a biomarker for atherosclerosis diagnosis.

The primary cells that contribute to atherosclerotic lesion formation are endothelial cells, vascular smooth muscle cells and macrophages. Overtake of oxidized low-density lipoprotein (ox-LDL) by macrophages induces the formation of lipid-laden foam cells, which can produce various of pro-inflammatory cytokines, such as matrixmetalloproteinases (MMPs), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor (TNF) α. The proliferation rate of vascular smooth muscle cells is low under normal circumstance. In response to the ox-LDL and activated macrophages, vascular smooth muscle can migrate to the artery intima, meanwhile proliferate and produce fibrous tissue, forming plaque. What’s more, this activated VSMC can also produce pro-inflammatory cytokines and engulf lipoproteins. In this study, we found that overexpression of lncRNA H19 in VSMC could lead to the increase in proliferation and decrease in apoptosis. Our result was similar with Li's findings. Li et al. reported that lncRNA H19/miR-675 could attenuate inflammation and apoptosis of cardiomyocyte in diabetic cardiomyopathy. This result indicated that lncRNA H19 may be the key factor in activating VSMC.

Studies have reported that lncRNA H19 could promote the invasion and migration ability of different kinds of tumors, but the effect of lncRNA H19 on the migration of VSMC remained unclear. To explore the role of lncRNA H19 on migration ability of VSMC, we detected the EMT marker of
LncRNA H19 promotes atherosclerosis by promoting the expression of p38 and p65

VSMC after lncRNA H19 was overexpressed. We found that E-cadherin was decreased while N-cadherin was increased significantly compared with the control group, indicating that lncRNA H19 could promote the migration ability of VSMC.

Both MAPK and NF-kB signaling pathways have been reported to be involved in regulation of atherosclerosis. Lee et al23 reported that lobastin could inhibit the expression of VCAM-1 (Vascular Cell Adhesion Molecular 1) on mouse vascular smooth muscle via MAPK and NF-kB signaling pathway, indicating that MAPK and NF-kB might regulate the adhesion ability of VSMC. Zhao et al25 also showed that MAPK and NF-kB signaling pathway could regulate the migration ability of mouse vascular smooth muscle through MAPK and NF-kB signaling pathway.

HSP60 (Heat Shock protein 60), which was closely associated with the pathogenesis of atherosclerosis, could stimulate the migration of VSMC via MAPK signaling pathway. Yu et al26 found that high level of glucose could promote the proliferation and migration of mouse vascular smooth muscle, via MAPK and NF-kB signaling pathway, and this effect could be suppressed by rutin. These studies showed that both MAPK and NF-kB signaling pathway were the key regulators in the formation and development of atherosclerosis, which could be treated as a potential target for treating atherosclerosis. To further explore the regulatory mechanism of lncRNA H19 in promoting atherosclerosis, we detected the expression of p38 and p65 after lncRNA H19 was overexpressed. We found that both p38 and p65 were highly expressed in VSMC. These findings indicated that lncRNA H19 might regulate atherosclerosis through MAPK and NF-kB signaling pathway.

Conclusions

We observed that lncRNA H19 was highly expressed in the serum of patients with atherosclerosis. H19 could also promote proliferation and reduce apoptosis of VSMC, suggesting that lncRNA H19 might regulate atherosclerosis via MAPK and NF-kB signaling pathway. Our study showed that lncRNA H19 might be a potential target for treating atherosclerosis.

Conflict of interest

The authors declare no conflicts of interest.

References


328

J.-X. Pan


