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HOTAIR alleviates ox-LDL-induced inflammatory response in Raw264.7 cells via inhibiting NF-κB pathway

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Abstract. – OBJECTIVE: To investigate the possible role of hox transcript antisense intergenic RNA (HOTAIR) in the pathogenesis of atherosclerosis and its underlying mechanism.

PATIENTS AND METHODS: The expression of HOTAIR in peripheral blood lymphocytes of atherosclerosis (AS) and healthy controls was detected by quantitative Real-time-polymerase chain reaction (qRT-PCR). *In vitro* AS model was established by ox-LDL induction in Raw264.7 cells. Viability of Raw264.7 cells after ox-LDL induction was detected by cell counting kit-8 (CCK-8) assay. Levels of TC (total cholesterol), TG (triglyceride), LDL-C (low density lipoprotein cholesterol) and HDL-C (high density lipoprotein cholesterol) in Raw264.7 cells were detected by enzyme-linked immunosorbent assay (ELISA). Overexpression plasmid of HOTAIR was constructed. Levels of TG, TC, LDL-C, and HDL were detected again after HOTAIR overexpression by ELISA. CD68+ cells and CD168+ cells in Raw264.7 cells were detected by flow cytometry. Protein expressions of pro-inflammatory and anti-inflammatory genes were detected by Western blot. Lipid metabolism in Raw264.7 cells was evaluated by oil red O staining and Western blot, respectively. Finally, rescue experiments were conducted to explore the specific mechanism of HOTAIR in regulating AS development.

RESULTS: HOTAIR was lowly expressed in peripheral blood lymphocytes of AS patients and Raw264.7 cells induced by ox-LDL. Overexpression of HOTAIR upregulated adipose genes (PPARα and CPT-1) and downregulated lipogenesis genes (SREBP-1c and ACS). Besides, overexpression of HOTAIR decreased expressions of pro-inflammatory cytokines (TNF-α and IL-1β), but increased expressions of anti-inflammatory cytokines (IL-4 and IL-10). In the in vitro AS model, FXR1 was remarkably downregulated in Raw264.7 cells. HOTAIR reduced inflammatory response via promoting FXR1 expression in Raw264.7 cells. Rescue experiments showed that the effect of HOTAIR on nuclear factor-kappa B (NF-κB) pathway was reversed by FXR1 knockdown.

CONCLUSIONS: We found that TAIR was lowly expressed in AS patients. Overexpression of HOTAIR can reduce the lipid accumulation and inhibit inflammatory response by suppressing FXR1 via NF-κB pathway.

Key Words:
Atherosclerosis, HOTAIR, FXR1, NF-κB pathway.

Introduction

Atherosclerosis (AS) is the most common and serious type of arterial pathological changes. AS often involves important organs, such as the heart and brain, seriously affecting life quality of affected population[1,2]. Its occurrence and development are closely related to the functional changes of macrophages and the proliferation of vascular smooth muscle cells (VSMCs). Current studies have suggested that AS is resulted from both environmental factors and genetic factors. In addition to traditional risk factors for AS, such as age, hypertension, dyslipidemia, smoking and diabetes, genetic factors also lead to the occurrence and development of AS. Genome-wide association studies (GWAS) and gene-based single nucleotide polymorphisms (SNPs) studies have identified AS-related genetic variations[3]. However, these genetic variations can only explain 6-10% of risk changes for AS[4,5]. With the active researches on epigenetics in recent years, the role of epigenetics in the occurrence and development of cardiovascular diseases has been well concerned. Long non-coding RNA (lncRNA) is a non-coding RNA with over 200 nucleotides in length[6]. Scholars have shown that lncRNAs participate in many

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biological activities, such as dose compensation effects, epigenetic regulation, cell cycle and cell differentiation[7, 8]. Hox transcript antisense intergenic RNA (HOTAIR) is one of the most studied lncRNAs with 2158 bp in length. HOTAIR exerts its biological function in a trans-silencing manner[9]. Great advances have already been made on HOTAIR function in breast cancer, colon cancer, adrenocortical carcinoma, pancreatic cancer, and other tumors[10-12]. However, the potential mechanism of HOTAIR in AS still remains unclear. Our study aims to explore the role of HOTAIR in the occurrence and progression of AS.

Patients and Methods

Lymphocyte Extraction

15 AS patients treated in the Tiantai People's Hospital of Zhejiang Province from 2015 to 2016 were selected. Meanwhile, 20 controls matched with age, sex, and underlying diseases were randomly selected. Blood sample of each subject was collected and incubated with ACD (acid citrate dextrose) anticoagulant at a ratio of 9: 1. Anticoagulant blood was then added in the upper layer of lymphocyte separation solution at a ratio of 1: 2, followed by centrifugation at 20°C, 400 g/min for 30 min. Subsequently, cells were resuspended in RPMI-1640 (Roswell Park Memorial Institute-1640), followed by centrifugation at 200 g/min for 10 min for three times. This study was approved by Hospital Ethic Committee and all subjects gave informed consent.

RNA Extraction And Quantitative Real-Time-Polymerase Chain Reaction (qrt-PCR)

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract total RNA for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was determined by a spectrophotometer (Hitachi, Tokyo, Japan). The expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. QRT-PCR reaction parameters were: 94°C for 15 s, 55°C for 30 s and 72°C for 30 s, for a total of 40 cycles. Primers used in the study were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5′-CACCCACTCCTCCACCTTTG-3′, R: 5′-CCACCACCCTGTTGCTGTAG-3′; HOTAIR: F: 5'-ATAGGCAAATGTCAGAGGGTT-3', R: 5'-ATTCTTAAATTGGGCTGGGTC-3'.

Cell Culture and ox-LDL Induction

Raw264.7 cells were obtained from ATCC (American Type Culture Collection) (Manassas, VA, USA). Cells were cultured in ECM (endothelial cell medium) (Enzo Life Sciences, Raamsdonksveer, The Netherlands) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a 5% CO_2 incubator at 37°C. After cell confluence was up to 80%, Raw264.7 cells were induced with 40 μg/mL ox-LDL for 24 h.

Cell Counting Kit-8 (CCK-8) Assay

Raw264.7 cells were seeded in the 96-well plates at a density of 2×10^3/mL. 10 μl of CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) were added in each well. After incubation for 2 h, the OD (optical density) value of each well was measured at the wavelength of 450 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

Blood Lipid Detection

Levels of TC (total cholesterol), TG (triglyceride), LDL-C (low density lipoprotein cholesterol) and HDL-C (high density lipoprotein cholesterol) in culture medium were detected by enzyme-linked immunosorbent assay (ELISA). Briefly, corresponding antibodies were diluted with coating buffer to 1-10 μg/mL for overnight incubation at 4°C. Each sample was incubated with 0.1 mL of antibody solution in a reaction well at 37°C for 1 h. Subsequently, 0.1 mL of diluted enzyme labeled antibody was added for another 1-h incubation, followed by color developing with TMB substrate solution. 0.05 mL of sulphuric acid was added for termination. The optical density was detected at the wavelength of 450 nm using ELISA detector.

Cell Transfection

After cell confluence was up to 60-80%, cell transfection was performed following the manufacturer's instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Overexpression plasmid of HOTAIR was: sense: 5'-CATGGATCCACATTCTGCCCTGATTTCCGGAACC-3'; antisense: 5'-ACTCTCGAGCCACCACACACACACAACCTACAC-3'.

Oil Red O Staining

Raw264.7 cells were fixed with formaldehyde for 10 min, immersed with 60% isopropanol and stained with oil red O for 10 min. The excess oil red O was washed and cells were re-stained with

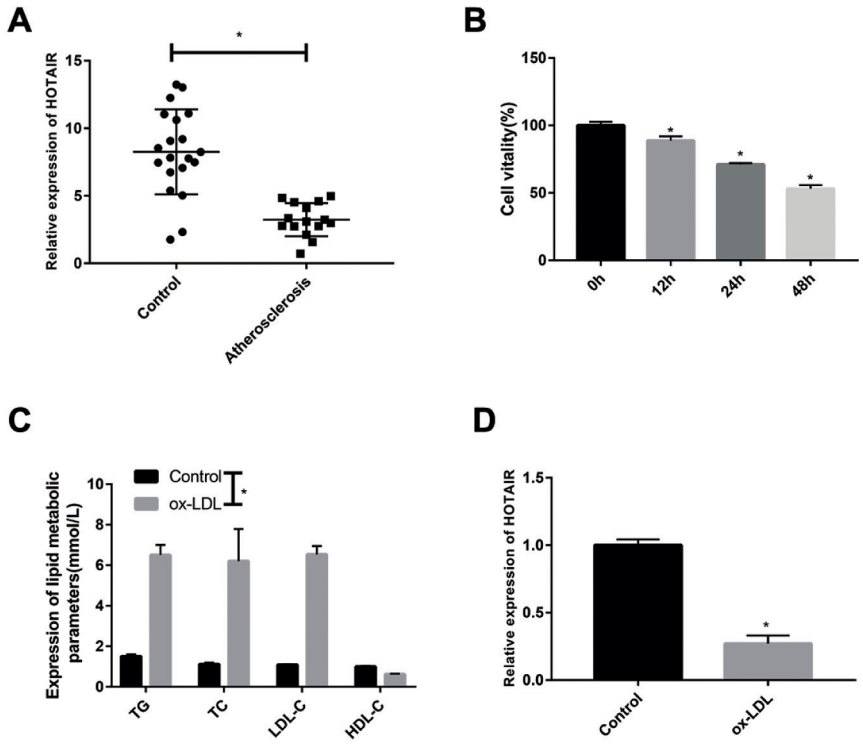

Figure 1. HOTAIR was lowly expressed in AS patients. ***A,*** QRT-PCR results showed that HOTAIR was lowly expressed in peripheral blood lymphocytes of AS patients than that of healthy controls. ***B,*** CCK-8 assay showed that viability of Raw264.7 cells was gradually decreased with the prolongation of ox-LDL induction. ***C,*** Elevated levels of TG, TC and LDL-C, as well as reduced HDL-C level were observed in Raw264.7 cells treated with ox-LDL for 24 h compared with those of controls. ***D,*** HOTAIR expression was downregulated in ox-LDL-induced Raw264.7 cells.

hematoxylin. Stained lipid droplets were captured using an optical microscope (Nikon, Tokyo, Japan).

Flow Cytometry

Third-passage Raw264.7 cells were collected and adjusted to a density of 3000-6000/μL. Cells were incubated with CD68 and CD168 labeled antibodies for 25 min in dark. After fixation with formaldehyde at 4°C overnight, positive expressions of CD68 and CD168 were detected by flow cytometry.

Western Blot

Raw264.7 cells were lysed using a cell lysis buffer, shaken on ice for 30 min, and centrifuged at 4°C, 14,000 ×g for 15 min. Total protein concentration was calculated by BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The extracted proteins were separated on a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel and subsequently transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures.

Statistical Analysis

SPSS (Statistical Product and Service Solutions) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Continuous variables were analyzed the *t*-test. $p<0.05$ was considered statistically significant.

Results

HOTAIR was Lowly Expressed in AS Patients

QRT-PCR results showed that HOTAIR was lowly expressed in peripheral blood lymphocytes of AS patients than that of healthy controls (Figure 1A). *In vitro* AS model was subsequently constructed by ox-LDL induction in Raw264.7 cells. CCK-8 assay showed that viability of Raw264.7 cells was gradually decreased with the prolongation of ox-LDL induction (Figure 1B). Induction of 40 μg/mL ox-LDL for 24 h was selected for the following experiments. Elevated levels of TG, TC and LDL-C, as well as reduced HDL-C level were

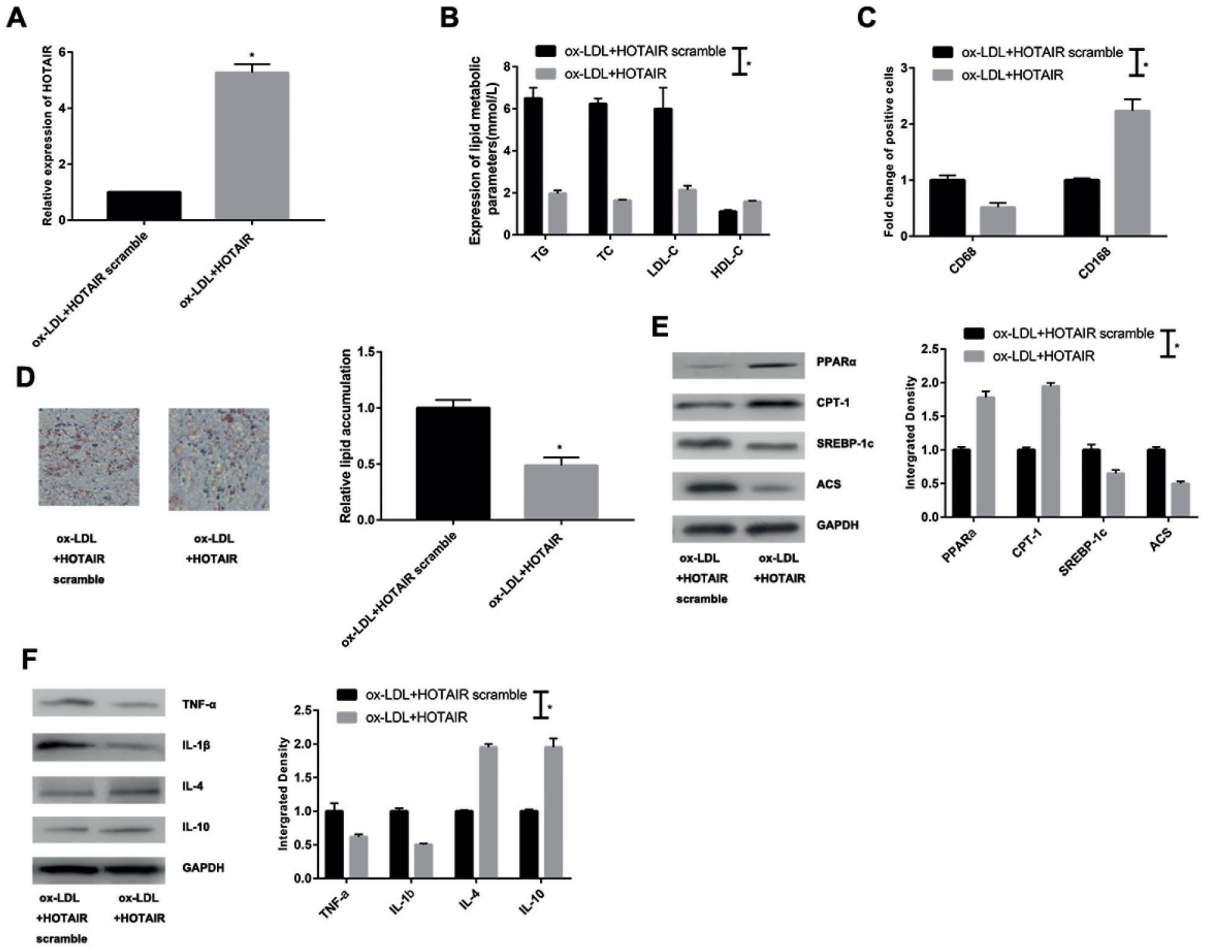

Figure 2. Overexpression of HOTAIR inhibited inflammatory response in Raw264.7 cells. ***A,*** Transfection efficacy of overexpression plasmid of HOTAIR was verified in Raw264.7 cells. ***B,*** ELISA data indicated that HOTAIR overexpression downregulated levels of TG, TC and LDL-C, whereas upregulated HDL-C level. ***C,*** Flow cytometry results indicated downregulated $CD68^+$ cells and upregulated $CD168^+$ cells after HOTAIR overexpression. ***D,*** Oil red O staining showed less stained lipid droplets after HOTAIR overexpression. ***E,*** HOTAIR overexpression remarkably upregulated PPARα and CPT-1, whereas downregulated SREBP-1c and ACS in Raw264.7 cells. ***F,*** Western blot results showed that pro-inflammatory factors were elevated and anti-inflammatory factors were decreased by transfection of HOTAIR overexpression plasmid.

observed in Raw264.7 cells treated with 40 μg/mL ox-LDL for 24 h compared with those of controls (Figure 1C). Furthermore, we detected HOTAIR expression in ox-LDL-induced Raw264.7 cells and found it was remarkably downregulated (Figure 1D). The above results indicated that the expression of HOTAIR is reduced during AS progression.

Overexpression of HOTAIR Inhibited Inflammatory Response in Raw264.7 Cells

Overexpression plasmid of HOTAIR was first constructed and its transfection efficacy was verified in Raw264.7 cells (Figure 2A). ELISA data indicated that HOTAIR overexpression downregulated levels of TG, TC and LDL-C, whereas upregulated HDL-C level (Figure 2B). Oil red O staining also showed less stained lipid droplets after HOTAIR overexpression (Figure 2D). Protein expressions of adipose genes and lipogenesis genes were detected by Western blot. HOTAIR overexpression remarkably upregulated PPARα and CPT-1, whereas downregulated SREBP-1c and ACS in Raw264.7 cells (Figure 2E). Since inflammation is a basic feature of AS, we further examined the effect of HOTAIR on the inflammatory response in Ox-LDL-induced macrophages. Flow cytometry results indicated downregulated $CD68^+$ cells and upregulated $CD168^+$ cells after HOTAIR overexpression (Figure 2C). Western blot results also showed that pro-inflammatory

factors were elevated and anti-inflammatory factors were decreased by transfection of HOTAIR overexpression plasmid (Figure 2F).

HOTAIR Promoted FXR1 Expression

FXR1 can affect the plasma levels of HDL, LDL, and TG by regulating genes related to lipid metabolism. To further explain the mechanism of HOTAIR in the involvement of AS, we found that FXR1 expression was remarkably reduced after ox-LDL treatment in Raw264.7 cells (Figure 3A). The downregulated FXR1 expression was increased after HOTAIR overexpression (Figure 3B). Subsequently, FXR1 siRNA was constructed to carry out the rescue experiments and its transfection efficacy was verified (Figure 3C). We found that FXR1 knockdown resulted in upregulated TNF-α and IL-1β, as well as downregulated IL-4 and IL-10 (Figure 3D). These results indicated that HOTAIR can reduce the inflammatory response by promoting FXR1 expression.

HOTAIR Inhibited NF-κB Pathway

NF-κB is involved in many significant physiological responses. Therefore, we detected the expressions of p-p65, p65, p-IκBα and IκBα after overexpression of HOTAIR. The results showed that HOTAIR can inhibit the activation of NF-κB pathway (Figure 4A). To observe whether FXR1 is involved in the regulation of NF-κB pathway by HOTAIR, we co-transfected FXR1 siRNA and HOTAIR overexpression plasmid in Raw264.7 cells. The results showed that FXR1 knockdown can reverse the inhibited NF-κB pathway induced by HOTAIR overexpression (Figure 4B). These results indicated that HOTAIR can inhibit NF-κB pathway by regulating FXR1.

Discussion

Atherosclerosis (AS) is a disease in which the inside of an artery narrows due to the buildup of

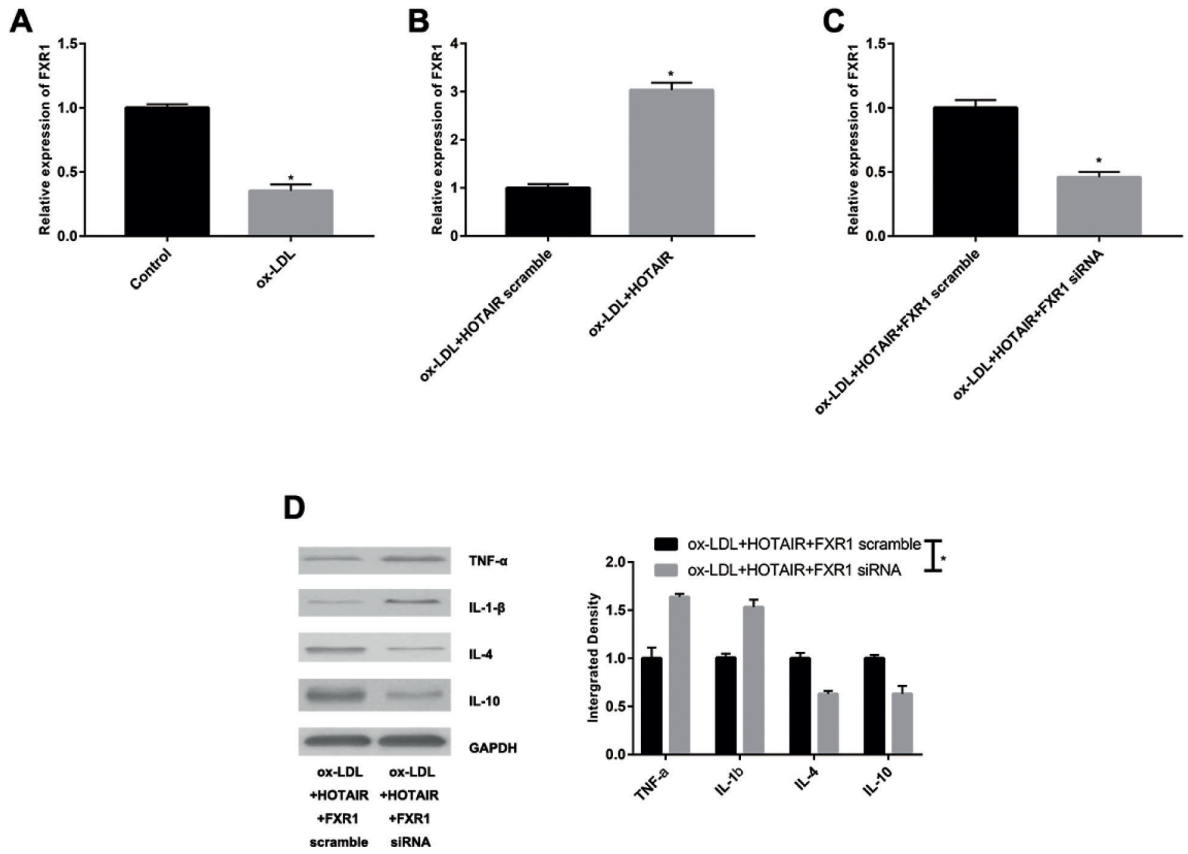

Figure 3. HOTAIR promoted FXR1 expression. ***A,*** FXR1 expression was remarkably reduced after ox-LDL treatment in Raw264.7 cells. ***B,*** The downregulated FXR1 expression was increased after HOTAIR overexpression. ***C,*** Transfection efficacy of FXR1 siRNA was verified. ***D,*** FXR1 knockdown resulted in upregulated TNF-α and IL-1β, as well as downregulated IL-4 and IL-10.

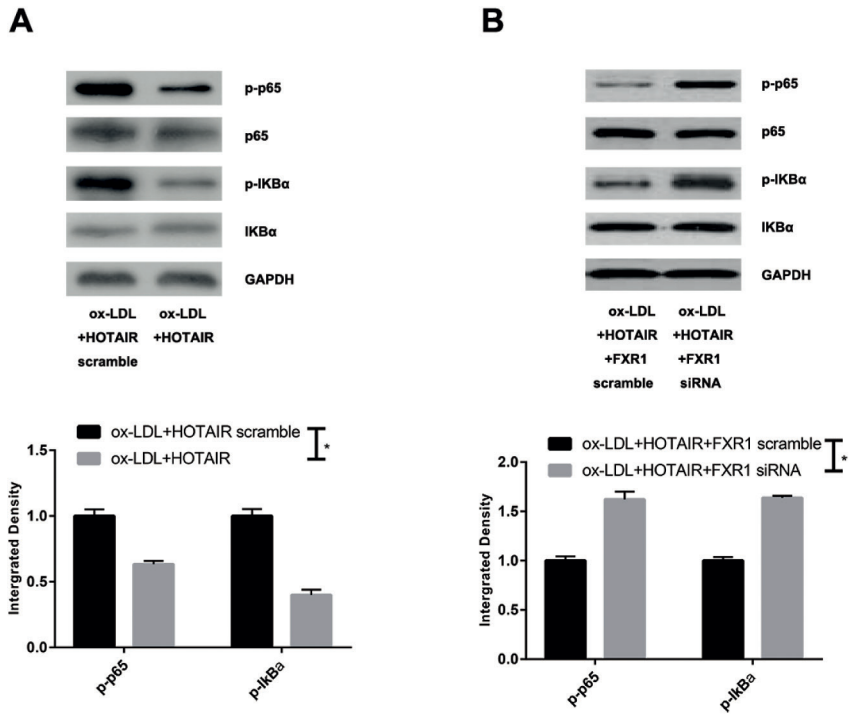

Figure 4. HOTAIR inhibited NF-κB pathway. ***A,*** HOTAIR can inhibit the activation of NF-κB pathway. ***B,*** FXR1 knockdown can reverse the inhibition of NF-κB pathway induced by HOTAIR overexpression.

plaque. Research on the pathogenesis of AS has been a hot topic in the study of cardiovascular diseases. It is believed that lipid infiltration, proliferation of smooth muscle cells and the thrombosis may explain for AS development. The comprehensive mechanism of AS is still not fully elucidated. Human Genome Project revealed that only about 2% of the genes express proteins. The majority of genes do not express proteins, which are non-coding RNAs (ncRNAs)[13]. Based on nucleotide sequence lengths and their biological functions, ncRNAs are divided into small interfering RNAs (siRNAs), microRNAs (miRNAs), PIWI-interacting RNAs, and lncRNAs[14]. HOTAIR was the first lncRNA to be found to have trans-transcriptional regulation that was only expressed in mammals. Human HOTAIR has a full length of 2,158 base pairs and is located between HOXC11 and HOXC12 on chromosome 12q13.13 with 6 exons[15]. Scholars[16-18] have shown that HOTAIR regulates the proliferation and apoptosis of breast cancer, liver cancer, esophageal cancer, lung cancer and other cancer cells *in vitro*. In this study, HOTAIR expression was lowly expressed in peripheral blood lymphocytes in AS patients. HOTAIR was also lowly expressed in Raw264.7 cells after ox-LDL induction. Ross[19] showed that AS is an inflammatory disease. AS is a chronic compensatory inflammatory reaction under the interaction of various factors, such as abnormal lipid metabolism, chronic inflammation, immune disorders, and inheritance. AS pathogenesis involves lipid deposition in vascular intima, endothelial cell damage, adhesion and infiltration of platelet, smooth muscle cell proliferation, collagen fibrosis, and foam cell formation[20]. The inflammatory response is a central link in plaque formation and instability. Overexpression of HOTAIR reduced lipid metabolism in ox-LDL-induced macrophages. It also alleviated inflammation *via* regulating expression levels of inflammatory factors. We proposed that HOTAIR may serve as a target drug for preventing and treating AS. FXR1 is a member of nuclear receptor superfamily, and is expressed in the liver, kidney, adrenal gland and small intestine[21]. FXR1 is involved in lipid metabolism, glucose metabolism, inflammation, and tumor development. FXR1 can reduce the accumulation of neutral lipids in diabetic rats and downregulate blood lipid levels. Relative genes that are regulated by FXR1 could reduce bile acid synthesis. Besides, FXR1 participates in

the regulation of cholesterol metabolism by inhibiting CYP7A1 activity[22]. In the present work, FXR1 was lowly expressed in the *in vitro* AS model. FXR1 was negatively regulated by HOTAIR, thereby decreasing the inflammatory response in Raw264.7 cells. Transcription factors of the NF-κB family participate in many key physiological responses, such as inflammatory responses, cell proliferation, differentiation, adhesion, and apoptosis[23]. In the process of AS, NF-κB pathway has been shown to be closely related to sterility inflammatory immune response[24]. The mammalian NF-κB family consists of five members, including p65, Re1B, c-Rel, p50, and p52. They are capable of activating target gene expressions *via* binding to κB enhancers[25]. In this study, HOTAIR inhibited the activation of NF-κB pathway, which could be reversed by FXR1 knockdown.

Conclusions

We found that HOTAIR was lowly expressed in AS patients. Overexpression of HOTAIR can reduce the lipid accumulation and inhibit inflammatory response by suppressing FXR1 *via* NF-κB pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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