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HOXA11-AS regulates diabetic arteriosclerosis-related inflammation via PI3K/AKT pathway

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Abstract. – OBJECTIVE: This study aims to explore whether homeobox A11 antisense RNA (HOXA11-AS) could regulate inflammation induced by diabetic arteriosclerosis (DAA) via PI3K/AKT pathway.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect expressions of HOXA11-AS and proinflammatory genes in carotid endarterectomy samples of symptomatic and asymptomatic atherosclerosis (AS) patients, diabetes mellitus (DM), and non-DM patients. The above-mentioned genes in DM animal model and non-DM animal model were also detected. We detected the expression of HOXA11-AS in vascular smooth muscle cells (VSMCs) treated with platelet-derived growth factor (PDGF) or PDGF inhibitor imatinib, respectively. Subsequently, we applied cell transfection technology to interfere with the expression of HOXA11-AS in VSMCs. In vascular endothelial cells (VECs) and VSMCs, we detected the effect of HOXA11-AS on the expressions of genes related to the proliferation, migration, and cell cycle. Then, VSMCs were treated with tumor necrosis factor-α (TNF-α), and the expression of HOXA11-AS was examined in VSMCs. The effect of HOXA11-AS on TNF-α-induced inflammation in VSMCs was detected as well. Finally, we analyzed the effect of HOXA11-AS on PDGF-induced activation of PI3K/AKT pathway in VSMCs and VECs.

RESULTS: HOXA11-AS expression was markedly increased in carotid endarterectomy specimens of symptomatic AS patients compared to that of asymptomatic AS patients. Expression levels of HOXA11-AS and pro-inflammatory genes were significantly elevated in carotid endarterectomy specimens of DM patients. Similarly, HOXA11-AS expression was also significantly increased in carotid arteries of DM mice compared with that of non-DM mice. PDGF could upregulate HOXA11-AS expression in VSMCs, which was reversed by PDGF inhibitor imatinib. HOXA11-AS knockdown could reduce the expressions of the proliferation-associated gene (PCNA) and the cycle-related genes (p21, p53), and also inhibited the proliferation and migration of VSMCs induced by PDGF. HOXA11-AS was upregulated by TNF-α. HOXA11-AS knockdown remarkably downregulated expressions of inflammation-related genes in VSMCs induced by TNF-α. In VECs, low expression of HOXA11-AS can inhibit the expression of TNF-α-induced pro-inflammatory genes and PDGF-induced vascular inflammation-related genes. Low expression of HOXA11-AS inhibited PDGF-induced activation of PI3K/AKT pathway in VSMCs and VECs.

CONCLUSIONS: HOXA11-AS may participate in DAA by activating the PI3K/AKT pathway to regulate inflammation in VSMCs and VECs.

Key Words:
XA11-AS, PI3K/AKT, DAA, VSMCs, VECs.

Introduction

The proportion of DM patients is increasing year by year with the rapid economic development and aging of the population. As time goes

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by, hyperglycaemia and hyperglycaemia-induced complications pose serious medical burdens, such as diabetic arteriosclerosis (DAA), atherosclerosis (AS) and cardiomyopathy. Studies have shown that the incidences of cardio-cerebrovascular diseases in DM patients are significantly higher than those of non-DM patients[1]. DAA is a major manifestation of chronic macroangiopathy in DM, which is an important cause of death and disability in DM. DAA pathogenesis is complex, includes metabolic factors, high glucose oxidation, endothelial dysfunction, inflammation, and prethrombotic state. VECs and VSMCs are the major cells for blood vessels. The proliferation of VECs and VSMCs may lead to vascular remodeling, narrowing of the lumen, and dysfunction of vasomotor regulation, which are the main pathological changes of AS. In the inflammatory pathogenesis of DM and AS, inflammatory factors are vital for affecting the occurrence and progression[2]. Injury triggers VECs to rapidly attract leukocytes to accumulate at inflammatory sites. Monocytes are subsequently adsorbed in the surface of blood vessels through adhesion molecules ICAM-1 and VCAM-1, making monocytes unable to follow blood flow in blood vessels[3,4]. Mononuclear cells adhere to the intimal surface of the blood vessels form macrophages, and then phagocytize large amounts of deposited lipoproteins to produce macrophage foam cells. Subsequently, pro-inflammatory cytokines IL-6 and tumor necrosis factor tumor necrosis factor-α (TNF-α) are secreted, thus recruiting more immunocytes[5,6].

Phosphatidylinositol 3-kinases (PI3K) signaling pathway and its downstream molecule protein kinase B (PKB/AKT) exert essential roles in cell growth, proliferation, survival, migration, cytoskeletal reorganization, inflammation, apoptosis, and other biological processes. Matsuura et al[7] have indicated that the PI3K/AKT pathway is associated with endothelial-mediated relaxation and contractile function. PI3K/AKT pathway is involved in the formation of AS through pathogenesis of neovascularization, accumulation of inflammatory cells, dysfunction of VSMCs, and promotion of vasoconstriction. Vascular remodeling promotes the occurrence and development of AS[8]. Therefore, effective inhibition of PI3K/AKT signaling pathway can alleviate the progression of cardiovascular disease[8,9].

Long non-coding RNA (LncRNA) is a non-coding RNA with 200 nt in length. LncRNA is involved in the gene expression regulation via transcriptional silencing, transcriptional activation, chromatin remodeling, and histone modification[10]. Homeobox A11 antisense RNA (HOXA11-AS) locates on the antisense DNA strand of the HOXA11-AS gene with 5.1 kb in length[11]. HOXA11-AS is differentially expressed in gastric cancer[12], rectal cancer[13], and cervical cancer[14]. Researches have found that HOXA11-AS is associated with cell cycle and tumor grade of gliomas, leading to poor prognosis[15]. HOXA11-AS may participate in the development of cervical cancer by acting on the HOXAA gene[16]. The differential expression of HOXA11-AS is related to metastasis, invasion, staging, and prognosis of human ovarian cancer and lung adenocarcinoma[17,18]. However, there were few reports about the differential expression of HOXA11-AS in DAA. We examined the differential expression of HOXA11-AS in symptomatic and asymptomatic AS patients, DM and non-DM patients, and carotid arteries of DM mouse and non-DM mouse. The biological function of HOXA11-AS in DAA-induced inflammation was further explored, so as to provide a new suggestion for treating DAA.

Patients and Methods

General Information

The study was approved by the Yantai Affiliated Hospital of Binzhou Medical University Medical Ethics Committee. The informed consent was obtained from all patients. Tissue samples were collected from atherosclerosis and DM patients who undergone carotid endarterectomy in Yantai Affiliated Hospital of Binzhou Medical University from April 2016 to December 2017. Tissue samples were stored in liquid nitrogen.

Cell Culture

Primary mouse VSMCs were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 25 mM glucose and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), whereas primary VSMCs in the experimental group were cultured in DMEM supplemented with 25 mM glucose and 1% FBS. Primary mouse VECs were cultured in DMEM containing 10% FBS, endothelial cell growth supplements, heparin, and 5 mM sodium gluconate. After serum starvation for 24 h, cells were treated with TNF-α (1 ng/mL) or platelet-derived growth factor (PDGF) (10 ng/mL), respectively. All experiments were repeated three times.

Cell Transfection

The lentiviral plasmid vector LV-shHOXA11-AS containing the shHOXA11-AS cDNA sequence was constructed, and the negative control LV-Vector was constructed by GenePharma (Shanghai, China). VECs and VSMCs were digested with 0.25% trypsin and plated in 6-well plates at a density of 4×10^5 cells per well. After 24 h, fresh media containing Polybrene (6 μg/mL) and virus solution was replaced. 24 h later, the infected cells were incubated in fresh medium without virus solution and Polybrene. VECs and VSMCs were transfected with LV-shHOXA11-AS or LV-Vector, respectively, followed by TNF-α induction.

Construction of DM Mouse Model

The experimental ApoE$^{-/-}$ mice were obtained from the Model Animal Research Center of Nanjing University. 16 ApoE$^{-/-}$ mice were randomly divided into experimental group and control group. Mice in the experimental group received an intraperitoneal injection of 80 mg/kg/d sodium citrate buffer containing streptozotocin. 10% high-sugar syrup was given to mice in the experimental group after injection. Mice in the control group also received an intraperitoneal injection of 80 mg/kg/d sodium citrate buffer containing streptozotocin. However, they were fed with normal water after injection. After intraperitoneal injection of streptozotocin for 6 days, 10% high-sugar syrup was given to mice for another 7 days. Consequently, mice developed symptoms such as polyuria and weight loss. Construction of DM mouse model was considered to be successful when blood sugar level was higher than 300 mg/dl. Animal experimental protocols were approved by the Binzhou Medical University Ethics Committee.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using the spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was calculated using the $2^{-\Delta Ct}$ method. QRT-PCR reaction conditions were 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. Primers used in the study were as follows: β-actin, forward: 5'-CTGAAGTACCCCATTGAACATGGC-3', reverse: 5'-CAGAGCAGTAATCTCCTTCTGCAT-3'; ICAM1, forward: 5'-GAACCAGAGCCTAGGAGAC-3', reverse: 5'-TCCAGGAACGGATGAACGA-5'; VCAM1, forward: 5'-CGGATTGCTGCTCAGATTG-3', reverse: 5'-AGTGTCGGGTACTGTGAT-3'; MCP1, forward: 5'-GCCCTAAGGTCTTCAGCACCTT-3', reverse: 5'-TGCTTGAGGTGGTTGTGGAA-3'; TNF-α, forward: 5'-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3', reverse: 5'-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3'; IL-1β, forward: 5'-GAAAGACGGCACACCCACCCT-3', reverse: 5'-GCTCTGCTTGTGAGGTGCTGATGTA-3'; IL-6, forward: 5'-GTGACAACCACGGCCTTCCCTACT-3', reverse: 5'-GGTAGCTATGGTACTCCA-3'; P21, forward: 5'-GTCGCTGTCTTGCACTCTGG-3', reverse: 5'-CCAATCTGCGCTTGGAGTGATA-3'.

CCK-8 (Cell Counting Kit-8) Assay

Transfected cells were seeded into 96-well plates at a density of 2×10^3/μL. 10 μL of a CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) was added in each well after cell culture for 12, 24, and 48 h, respectively. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Assay

The logarithmic growth phase cells were prepared into cell suspension at a density of 1×10^5/mL. 200 μL of cell suspension was added into the upper chamber and 500 μl of medium containing 10% FBS was added into the lower chamber, respectively. The cells were allowed to invade for 36 h at 37°C in a humidified incubator containing 5% CO_2. The un-penetrating cells in the upper chamber were gently wiped off with a cotton swab. The cells were fixed in 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 10 min. The number of cells in five randomly selected fields was captured under a microscope at a magnification of 100×. The average number was taken as the number of cells passing through the chamber in each group.

Western Blot

Cells were lysed with RIPA (radioimmunoprecipitation assay) lysis buffer in the presence of a protease inhibitor (Sigma-Aldrich, St. Louis, MO,

USA) to harvest total cellular protein. The protein concentration of each cell lysate was quantified using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein sample was loaded onto a 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel and then transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA) after being separated. After blocked with skim milk, membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C and then incubated with HRP (horseradish peroxidase) conjugated secondary antibody for 2-3 h at room temperature. Finally, an image of the protein band was captured by the Tanon detection system using enhanced chemiluminescence (ECL) reagent (Thermo, Waltham, MA, USA).

Statistical Analysis

All experiments were repeated 3 times. The experimental data were expressed as mean ± SD ($\bar{x} \pm s$). The experimental results were analyzed with standard *t*-test analysis and Graph Pad Prism software (La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

Results

HOXA11-AS Expression in AS

Initially, we detected HOXA11-AS expression in carotid artery samples of AS patients and DM patients using qRT-PCR. Compared with asymptomatic AS patients, carotid artery samples in symptomatic AS patients were significantly increased (Figure 1A). Similarly, HOXA11-AS expression in carotid endarterectomy samples

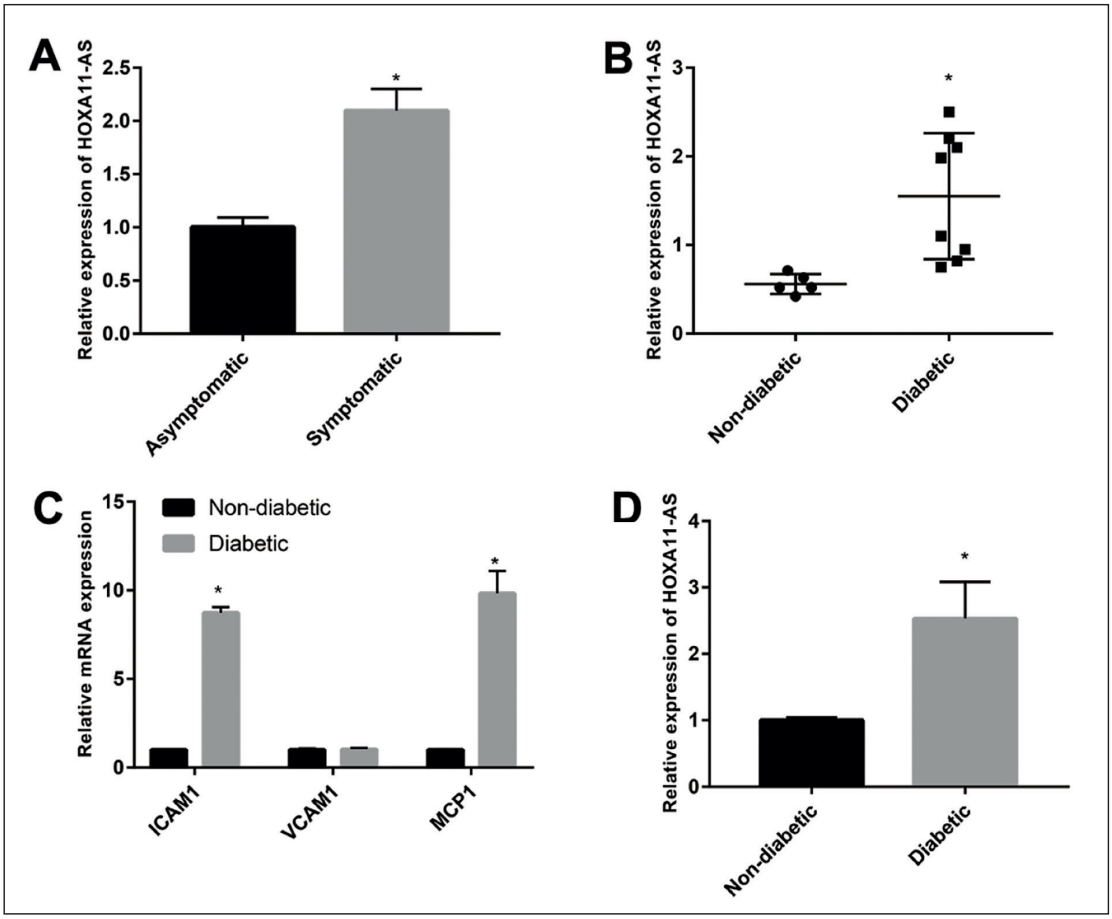

Figure 1. HOXA11-AS expression in AS. ***A,*** Compared with asymptomatic AS patients, carotid artery samples in symptomatic AS patients were significantly increased. ***B,*** HOXA11-AS expression in carotid artery samples of DM patients was remarkably higher than of non-DM patients. ***C,*** Expressions of pro-inflammatory genes (ICAM1, VCAM1 and MCP1) were significantly increased in DM patients compared with those of non-DM patients. ***D,*** Compared with non-DM mice, HOXA11-AS expression was remarkably increased.

of DM patients was remarkably higher than of non-DM patients (Figure 1B). Additionally, we examined the expression of pro-inflammatory genes in carotid endarterectomy samples in DM patients. The results showed that the expressions of pro-inflammatory genes (ICAM1, VCAM1, and MCP1) were significantly increased in DM patients compared with those of non-DM patients (Figure 1C). Subsequently, we established a DM mouse model. Similarly, compared with non-DM mice, HOXA11-AS expression was remarkably increased as well (Figure 1D).

HOXA11-AS Regulated Activation and Proliferation of VSMCs

To further investigate the effect of HOXA11-AS on the proliferation and migration of VSMCs, VSMCs were treated with pro-inflammatory factor PDGF, PDGF inhibitor Imatinib, or PDGF+imatinib, respectively. The results showed that after VSMCs were treated with PDGF at 10 ng/ml for 24 h, HOXA11-AS expression was remarkably up-regulated compared to that of the control group, while imatinib treatment reversed HOXA11-AS expression level to the normal one (Figure 2A). Furthermore, we constructed the lentiviral plasmid vector LV-shHOXA11-AS. QRT-PCR results showed that HOXA11-AS knockdown inhibited the expressions of the PDGF-induced proliferation-related gene (PCNA) and cell cycle-related genes (p21 and p53) (Figure 2B). CCK-8 results indicated that knockdown of HOXA11-AS inhibited proliferation of PDGF-induced VSMCs (Figure 2C). We further used transwell assays to determine the effect of HOXA11-AS on the migration of VSMCs. The number of penetrating cells and migrated VSMCs were decreased after HOXA11-AS knockdown (Figure 2D). Subsequently, after VSMCs were induced with 1 ng/ml TNF-α for 24 h, HOXA11-AS expression was upregulated (Figure 2E). However, low expression of HOXA11-AS inhibited the upregulated expressions of IL-6 and IL-1β in VSMCs induced by TNF-α (Figure 2F).

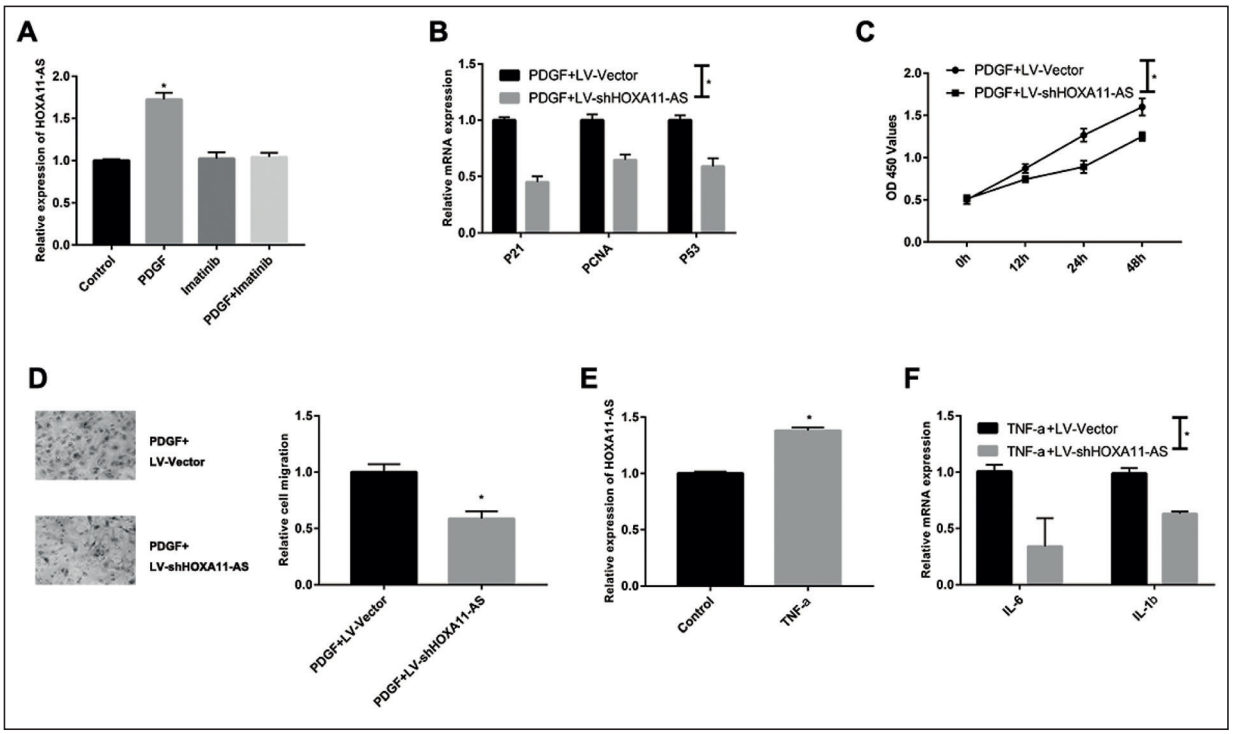

Figure 2. HOXA11-AS regulated activation and proliferation of VSMCs. ***A,*** After VSMCs were treated with PDGF at 10 ng/mL for 24 h, HOXA11-AS expression was remarkably up-regulated compared to that of the control group, while imatinib treatment reversed HOXA11-AS expression level to the normal one. ***B,*** QRT-PCR results showed that HOXA11-AS knockdown inhibited the expressions of the PDGF-induced proliferation-related gene (PCNA) and cell cycle-related genes (p21 and p53). ***C,*** CCK-8 results indicated that knockdown of HOXA11-AS inhibited proliferation of PDGF-induced VSMCs. ***D,*** The number of penetrating cells and migrated VSMCs were decreased after the HOXA11-AS knockdown. ***E,*** After VSMCs were induced with 1 ng/ml TNF-α for 24 h, HOXA11-AS expression was upregulated. ***F,*** Low expression of HOXA11-AS inhibited the upregulated expressions of IL-6 and IL-1β in VSMCs induced by TNF-α.

HOXA11-AS Regulates Activation and Proliferation of VECs

The role of HOXA11-AS in the proliferation and migration of VECs was further detected. VECs were first induced by 1 ng/ml TNF-α or 10 ng/ml PDGF for 24 h. HOXA11-AS knockdown reduced the expressions of pro-inflammatory genes MCP1, ICAM1, IL-6, and VCAM1 (Figure 3A). Besides, knockdown of HOXA11-AS also inhibited expressions of vascular inflammation-related genes MCP1 and IL-6 (Figure 3B). We also found that knockdown of HOXA11-AS suppressed PDGF-induced proliferation (Figure 3C) and migration (Figure 3D) of VECs.

Low Expression of HOXA11-AS Inhibited PI3K/AKT Pathway

To further clarify the effect of HOXA11-AS on the PI3K/AKT pathway, we analyzed the phosphorylation levels of PI3K and AKT in VSMCs and VECs by Western blot. The results showed that phosphorylation levels were markedly lower in VSMCs and VECs with lower expression of HOXA11-AS (Figure 4A and 4B). Our data demonstrated that knockdown of HOXA11-AS could inhibit the activation of the PI3K/AKT pathway in VSMCs and VECs.

Discussion

DM is a metabolic disease characterized by hyperglycemia due to defective insulin secretion or impaired insulin action. Persistent hyperglycemia and long-term metabolic disturbances could induce degeneration and dysfunction of systemic tissues and organs, particularly in the eyes, kidneys, cardiovascular and nervous system. DM-in-

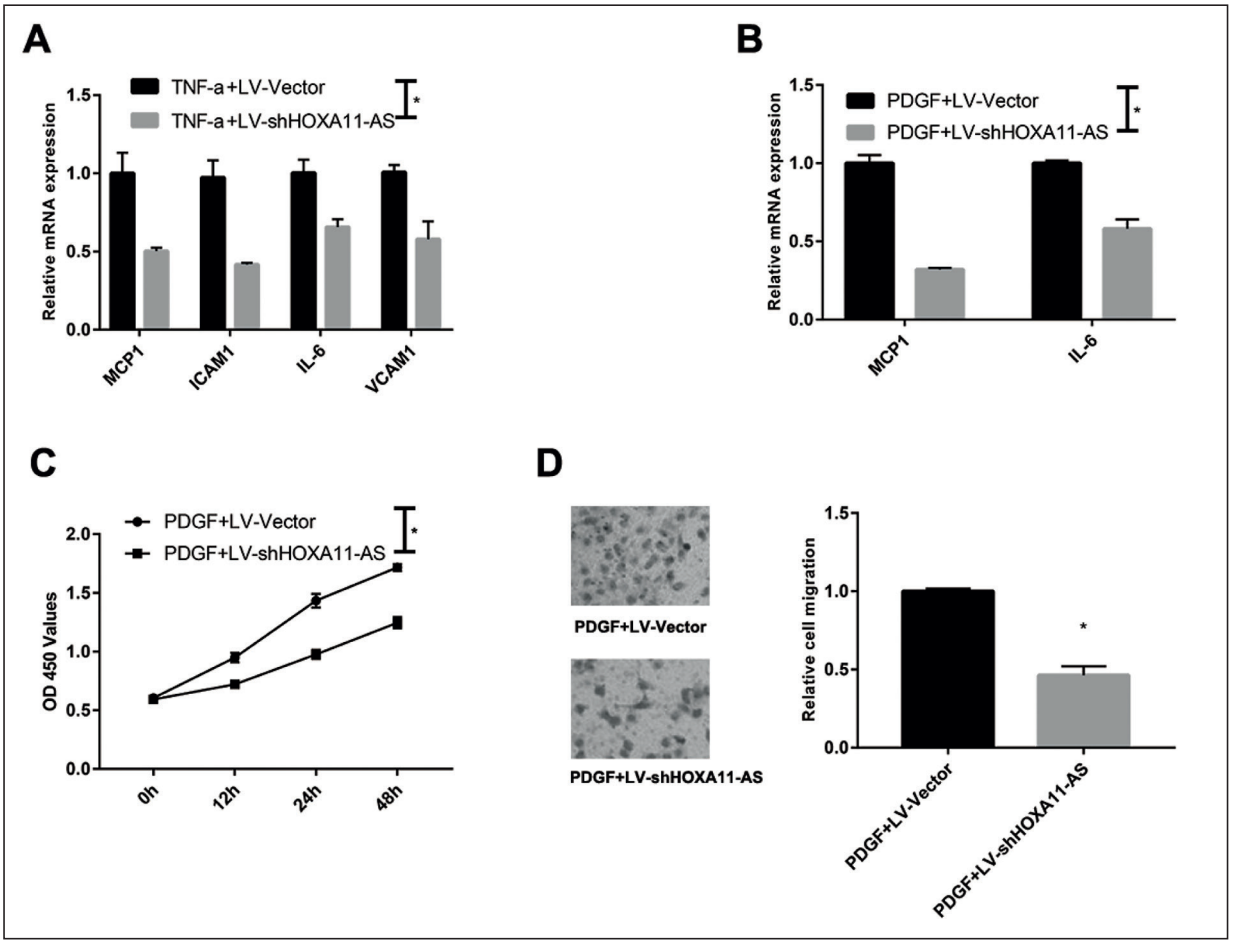

Figure 3. HOXA11-AS regulates activation and proliferation of VECs. ***A,*** HOXA11-AS knockdown reduced the expressions of pro-inflammatory genes MCP1, ICAM1, IL-6, and VCAM1. ***B,*** Knockdown of HOXA11-AS inhibited expressions of vascular inflammation-related genes MCP1 and IL-6. ***C, D,*** Knockdown of HOXA11-AS suppressed PDGF-induced proliferation and migration of VECs.

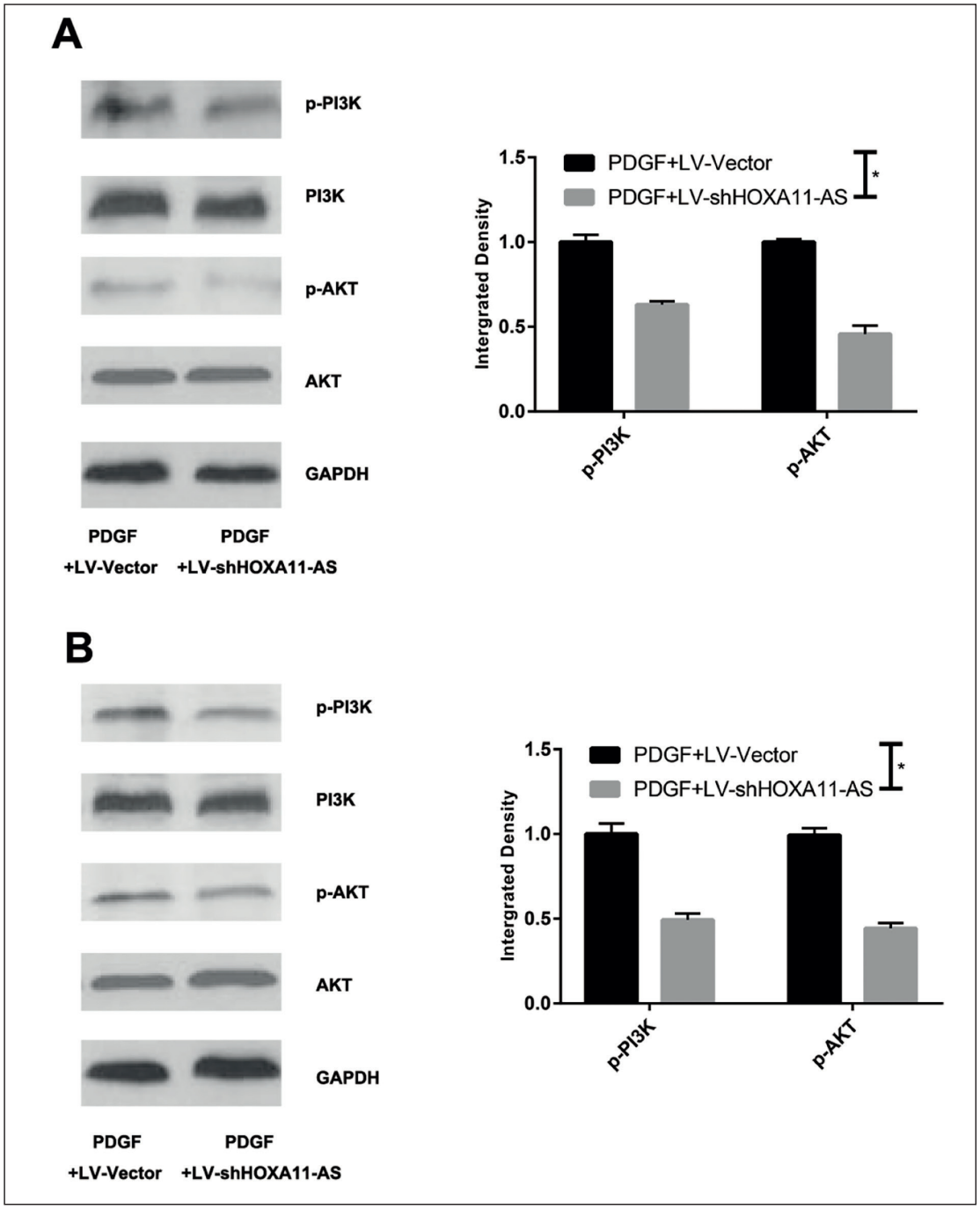

Figure 4. Low expression of HOXA11-AS inhibited the PI3K/AKT pathway. ***A, B,*** Phosphorylation levels were markedly lower in VSMCs and VECs with lower expression of HOXA11-AS.

duced chronic complications include macrovascular disease (e.g., cardiovascular, cerebrovascular and lower extremity macrovascular disease), microvascular disease (e.g., nephropathy and retinopathy) and neuropathy (e.g., systemic neuropathy and focal neuropathy)[19]. Among them, the macrovascular disease is the most common and the leading cause of death and disability in DM patients. The dysfunction of VECs is attributed to the early manifestation and initiation of diabetic vascular lesions. Long-term hyperglycemia would imbalance vascular function, resulting in AS, and further developing thrombotic lesions that endanger life[20]. Hyperglycemia, insulin resis-

tance and dyslipidemia are all risk factors for the VECs dysfunction in DM patients. VECs are the first barrier covering the surface of blood vessel walls and could directly sense this harmful stimulation in blood circulation, leading to dysfunction and apoptosis in VECs. Our observations suggested that endothelial dysfunction in type 2 diabetes is associated with the downregulation of the PI3K/AKT pathway, which is involved in multiple biological functions[21,22]. Activation of PI3K changes AKT conformation, leading to activation of AKT protein in VECs. At the same time, it inhibits apoptosis through phosphorylation of pro-apoptotic protein and stimulation of NO secretion. Therefore, PI3K becomes the core of the PI3K/AKT pathway. PI3K/AKT pathway can regulate the downstream eNOS, which is the key molecule regulating vascular function. It could produce NO to act on the vasodilatation and regulate vascular tension, so as to maintain the blood vessels smooth and blood supply to organs and tissues[23,24].

LncRNA is widely involved in almost all physiological and pathological processes of the body. It also has a close relationship with the occurrence and development of various clinical diseases including DM and AS. Relative studies have suggested that lncRNA-MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) is significantly upregulated in animal models of DM. Knockdown of lncRNA-MALAT1 reduces retinal inflammation in DM animal model and inhibits proliferation and migration of retinal endothelial cells[25]. The level of circulating lncRNA GAS5 correlates with the disease degree of DM[26]. Overexpression of lncRNA ANRI activates the NF-κB signaling pathway, upregulates VEGF expression, and promotes angiogenesis in a rat model of DM with cerebral infarction[27]. Serum level of lncRNA H19 is higher in DM patients than that of normal people. In HUVECs, overexpression of lncRNA H19 could advance the differentiation and inhibit the apoptosis of HUVECs. Potentially, it induces the progress of AS through MAPK and NF-κB signaling pathways[28]. When a vascular smooth muscle is stimulated with IL-1α and platelet-derived growth factor, the expression level of lncRNA SMILR is significantly increased. Knockdown of lncRNA SMILR could inhibit the differentiation of VSMCs and impair the occurrence of AS[29]. Downregulation of lncRNA HOXA11-AS can affect the proliferation and migration of trophoblast cells by regulating the expressions of RND3 and HOXA7 in pre-eclampsia[30]. Overexpression of lncRNA HOXA11-AS can prevent cell cycle, invasion, and metastasis of gastric cancer cells[31].

Here, we reported that lncRNA HOXA11-AS was upregulated in carotid endarterectomy samples of patients with symptomatic AS, DM, and DM mouse. The results showed that low expression of HOXA11-AS could inhibit the proliferation-associated gene PCNA and the cycle-related genes p21 and p53 in PDGF-induced VECs. Besides, HOXA11-AS knockdown resulted in a marked decrease in cell proliferation and migration. The low expression of HOXA11-AS could suppress TNF-α-induced inflammation via activating the PI3K/AKT pathway.

Conclusions

We showed that HOXA11-AS may participate in DAA by activating the PI3K/AKT pathway to regulate inflammation in VSMCs and VECs.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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