European Review for Medical and Pharmacological Sciences 2019; 23: 2223-2233

MiR-155-5p inhibits the proliferation and migration of VSMCs and HUVECs in atherosclerosis by targeting AKT1

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Abstract. – OBJECTIVE: MiR-155-5p has various biological cellular functions in diverse pathology, including cardiovascular disease. Nevertheless, the role of miR-155-5p in atherosclerosis is still not well known.

PATIENTS AND METHODS: The levels of miR-155-5p and AKT Serine/Threonine Kinase 1 (AKT1) in plasma samples from patients with atherosclerotic CAD were detected using quantitative Real-time PCR (qRT-PCR). Cell counting kit-8 (CCK-8) assay was used to analyze the proliferation of vascular smooth muscle cells (VSMCs) and human umbilical vein endothelial cells (HUVECs) *in vitro*. The migration of VSMCs and HUVECs was detected using wound healing assay. The invasion of VSMCs and HUVECs using was determined using the transwell invasion assay. The expression of AKT1 was measured using immunofluorescence staining analysis.

RESULTS: MiR-155-5p was down-regulated in patients with atherosclerotic CAD. Up-regulation of miR-155-5p inhibited the proliferation, migration and invasion of VSMCs and HUVECs. Bioinformatics analysis and luciferase reporter assay indicated that AKT1 was the direct target of miR-155-5p and miR-155-5p bound to the 3'-untranslated region (3'-UTR) of AKT1. The expression of AKT1 was reduced in cell that was transfected with miR-155-5p. Up-regulation of AKT1 rescued the suppressive effect of miR-155-5p on the growth, migration and invasion of VSMCs and HUVECs. Down-expression of AKT1 partially neutralized the impacts of miR-155-5p on the growth, invasion and migration of VSMCs and HUVECs. Finally, we found that AKT1 was over-regulated in plasma samples of patients with atherosclerotic CAD and its level was negative with the level of miR-155-5p.

CONCLUSIONS: Our study demonstrates that miR-155-5p suppresses the proliferation, migration and invasion of VSMCs and HUVECs through regulating AKT1, which provides the new insights into the precise role of miR-155-5p in atherosclerosis.

Key Words:
AKT1, Atherosclerosis, VSMCs, MiR-155-5p, HUVECs.

Introduction

Atherosclerosis, which is a multifactorial chronic disease, is characterized by the deposition of fibrous elements and lipids in the aorta[1-3]. One of the main components of the arterial wall is vascular smooth muscle cells (VSMCs), which have a variety of physical functions[4-6]. Recent investigations have demonstrated that most of the atherosclerotic lesions are derived from smooth muscle cells (SMC) and abnormal proliferation and migration of VSMCs promotes the development, expansion, and reorganization of the atherosclerotic lesions[7]. Hence, VSMCs play crucial roles in the progression of atherosclerosis. In addition, endothelial cells (ECs) play crucial roles in maintaining the homeostasis of the vascular system[8]. Current evidences have indicated that the initial qualitative change in the development of atherosclerosis is the injury of the endothelial cells, which line the inner wall of the arteries[9,10]. The microRNAs (miRNAs) are an extensive class of small noncoding RNAs (18 to 25 nucleotides) with probable roles in the regulation of gene expression. MiRNAs bind with the 3'-untranslated regions (3'-UTR) of target genes and reduce the expression of gene through degradation or translation of target gene[11]. The dysregulation of miRNAs in blood circulation and tissue have been proved to be biomarkers for the clinical diagnosis and prognosis in patient with atherosclerosis[12-15]. An extensive body of research has demonstrated that miRNAs regulate a lot of biological procedures, including cell differentiation and proliferation[16,17].

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Recently, several investigations have indicated that the relationship between the VSMC functions with the dysregulation of miRNA during the progression of atherosclerosis[18]. For example, miR-499a-3p and miR-135b-5p have been confirmed to be biomarkers of atherosclerosis and play crucial roles in regulating the migration and proliferation of VSMC via suppressing the expression of myocyte enhancer factor 2C (MEF2C)[19]. In addition, miR-145 regulates the phenotypic conversion of VSMCs in atherosclerosis[20,21]. In addition, miRNA-497 inhibits the proliferation of HUVECs and induces the apoptosis through targeting cyclin D2 protein and regulating the Bcl-2/Bax-Caspase9-Caspase3 signaling pathway in atherosclerosis[22]. Previous studies have shown that overexpression of miR-155-5p inhibits the proliferation and migration of interleukin IL-13 (IL-13) induced human bronchial smooth muscle cells by suppressing TGF-β-activated kinase 1/MAP3K7-binding protein 2 (TAB2)[23]. AKT Serine/Threonine Kinase 1 (AKT1) is a major regulator of the survival of VSMCs *in vivo* during vessel remodeling and atherogenesis through inhibition of Forkhead Box O3 (FoxO3a) and its downstream genes, including apoptotic protease activating factor 1 (Apaf-1)[24,25]. Down-regulation of AKT1 inhibits the migration and survival of VSMCs and improves the features of plaque weak and heart insufficiency during atherosclerosis[26]. All these investigations demonstrate that AKT1 is essential for the proliferation and migration of VSMCs[27,28]. Nevertheless, the potential role of miR-155-5p in the cellular processes of HUVECs and VSMCs has not yet well investigated. Herein, we analyzed the levels of miR-155-5p in the plasma of normal control and patients with atherosclerotic CAD. In addition, we assessed the effects of miR-155-5p on the proliferation, invasion and migration of VSMCs and HUVECs.

Patients and Methods

Patients

A total of 67 patients with selective coronary angiography (CAG) whom were treated at the First Affiliated Hospital of Soochow University were participated in the study. Patients with liver disease, renal failure, inflammatory diseases, valvular heart disease, or autoimmune disease were excluded. Isolated plasma samples were stored at -80°C until investigation. The study conforms to the Code of Ethics of the World Medical Association (Declaration of Helsinki) printed in the British Medical Journal (18 July 1964). This study was approved by Ethics Committee of the First Affiliated Hospital of Soochow University. Informed consent forms were signed by all patients before study.

Cell Culture

Human vascular smooth muscle cells (VSMCs), human umbilical vein endothelial cells (HUVECs) and HEK-293T cell were purchased from the Institute of Culture Collection of the Chinese Academy of Sciences (Shanghai, China). VSMCs was cultured in Medium 199 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Wisent, Quebec, Canada), 100 μg/ml penicillin, and 100 μg/ml streptomycin. HEK-293T and HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Wisent, Quebec, Canada), 100 Ul penicillin, and 100 μg/ml streptomycin. All cells were cultured in a humidified 37°C incubator containing 5% CO_2.

Cell Transfection

The miR-155-5p mimic and mimic control (miR-NC) were synthesized by SONGON BIOTECH (Shanghai, China). shRNA targeting AKT1 (shAKT1) and shRNA control (shCon) were synthesized by SONGON BIOTECH (Shanghai, China). The pcDNA3.1-AKT1 overexpression plasmid was constructed by PCR amplification of human AKT1 using cDNA as a template and subcloning into vector pcDNA3.1. Plasmids or miRNA was transfected into cells using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real-time PCR Assay

The plasma RNA was extracted using the Blood (Serum, Plasma) microRNA Mini Kit (Qiagen, Hilden, Germany). The total cellular was extracted from cells using TRIzol reagent (Sangon, Shanghai, China). For the analysis of miR-155-5p, reverse transcription of total RNA into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA, USA) and miR-155-5p specific stem-loop primers. For relative quantification of miR-155-5p, a Perfect Real Time SYBR Premix Ex Taq Kit (TaKaRa, Otsu, Shiga, Japan) was used for quantitative Real-time PCR. cDNA synthesis was performed using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. GAPDH

and U6 were an internal control. The primers were as follows: miR-155-5p (forward primer: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCTGAGA-3'; reserve primer: 5'-ACACTCCAGCTGTAAACATCCTACACTCT-3'), U6 (forward primer: 5'-AAAGCAAATCATCGGACGACC-3'; reverse primer: 5'-GTACAACACATTGTTTCCTCGGA-3'), GAPDH (forward primer: 5'-TGTGGGCATCAATGGATTTGG-3'; reverse primer: 5'-ACACCATGTATTCCGGGTCAAT-3'), AKT1 (forward primer: 5'-CCTCCACGACATCGCACTG-3'; reverse primer: 5'-TCACAAAGAGCCCTCCATTATCA-3'); ARID2 (forward primer: 5'-ACCAGTCTAAGGGATTAGGACC-3'; reverse primer: 5'-TGCTGGGACTATTCGGCTGA-3'); ETS1 (forward primer: 5'-GATAGTTGTGATCGCCTCACC-3'; reverse primer: 5'-GTCCTCTGAGTCGAAGCTGTC-3'); BACH1 (forward primer: 5'-TCTGAGTGAGAACTCGGTTTTTG-3'; reverse primer: 5'-CGCTGGTCATTAAGGCTGAGTAA-3'); HBP1 (forward primer: 5'-AAGCAGCCCTACAGTACAAGG-3'; reverse primer: 5'-GTGTGCTGGAGGGTCTGAAAC-3'); RAB5C (forward primer: 5'-CCGCTTTGTCAAGGGACAGTT-3'; reverse primer: 5'-AGGCTGTGATACCGCTCCT-3'); RCN2 (forward primer: 5'-TGGACTCAGATGGCTTTCTCA-3'; reverse primer: 5'-GACCTGAATCCTGGTTAGCTTTT-3'); MORC3 (forward primer: 5'-TCCTGATGTGAACGCTAAACAAA-3'; reverse primer: 5'-GAACCCGACTTGAAGCCATTC-3'); GPM6B (forward primer: 5'-CCGGGGTGGCCTTATTCTG-3'; reverse primer: 5'-GGTGGAGAAGTGTTGCTCAAGA-3'); SMAD2 (forward primer: 5'-CGTCCATCTTGCCATTCACG-3'; reverse primer: 5'-CTCAAGCTCATCTAATCGTCCTG-3'); WBP1L (forward primer: 5'-GCAGCGGCAACATGAAATCAA-3'; reverse primer: 5'-GTTGGAGGTCGGTTCACCAC-3'). The comparative cycle threshold (Ct) method was selected to detect the level by calculating the 2(-ΔΔCt).

Luciferase Reporter Assay

The 3'-UTR fragment of the AKT1 gene containing the binding site of miR-155-5p was amplified from human genomic DNA by PCR and cloned into the luciferase reporter vector pGL3-promoter (Promega, Madison, WI, USA) to construct the pGL3-AKT1-3'-UTR wild type (pGL3-AKT1-3'-UTR-WT) vector. A mutation of the AKT1 3'-UTR sequence was designated and introduced into pGL3-AKT1-3'-UTR-WT to generate recombinant plasmid pGL3-AKT1-3'-UTR mutant (pGL3-AKT1-3'-UTR-MUT) using a Quick-change Site-Directed Mutagenesis Kit (Stratagene, Lo Jolla, CA, USA) according to the manufacturer's protocol. HEK-293T cell was cotransfected with miR-155-5p and luciferase reporter plasmid using the LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Cell Counting Kit-8 (CCK-8) Assay

The proliferation of VSMCs and HUVECs at different time points (24 h, 48 h, 72 h or 96 h) was determined using Cell Counting Kit-8 (CCK-8) (Beyotime, Nanjing, Jiangsu, China). Cell (2×103) was seeded into 96-well plates. Subsequently, 20 µl of CCK-8 solution was added to each well at 24 h, 48 h, or 72 h. The cell was cultured for 1 h at 37°C. Finally, the absorbance at 450 nm was measured with a microplate reader.

Migration Assay

Cells were seeded into a 6-well plate. After 24 h, the monolayer was scratched using a sterile 100 µl tip. The photos were taken at 0 h and 24 h using a digital camera system (Olympus, Tokyo, Japan). Scratch healing rate = (scratch width 0 h − scratch width 24 h)/ scratch width 0 h × 100%.

Invasion Assay

Transwell chambers (24-well Transwell chambers, 8-µm pore size; Corning, Inc., Corning, NY, USA) were used for invasion assay. 200 µl cell suspension (2 × 103) was seeded into upper chambers and 600 µl medium containing 10% fetal bovine serum (FBS) was added into the lower chamber. After 24 h, the cells that invaded the lower surface of the chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of invaded cell was counted from five random fields by bright field microscopy.

Enzyme Linked Immunosorbent Assay (ELISA)

The level of AKT1 in plasma was evaluated by human AKT1-specific sandwich ELISA kit (Abcam, Cambridge, MA, USA) according to the kit instructions.

Immunofluorescence

Cells on glass coverslips were fixed by pre-cold acetone and then rinsed three times with PBS. The cells were permeabilized in 0.1% Triton X-100 and incubated with 1% BSA/PBS to block nonspeci-

fic binding. Subsequently, the cells were immunostained by incubating with rabbit monoclonal antibody against AKT1 (diluted 1:500, Epitomics, Burlingame, CA, USA) overnight at 4°C. After being washed with PBS, cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (diluted 1:60, Boster Biotechnology, Wuhan, Hubei, China). Nuclei were counterstained with DAPI (Biotime Biotech, Haimen, Jiangsu, China). Images were taken and analyzed using the ZEN 2011 imaging software on a Zeiss invert microscope (CarlZeiss, Hallbergnoos, Germany).

Statistical Analysis

All the experiments were repeated three times and analyzed with GraphPad Prism (Prism 5.0, GraphPad Software, La Jolla, CA, USA). The data were presented as mean ± SD. Differences in the results of two groups were evaluated using either two-tailed Student's t-test or one-way ANOVA followed by post hoc Dunnett's test. The differences with $p < 0.05$ were considered statistically significant.

Results

Up-regulation of MiR-155-5p Inhibits the Proliferation of VSMCs

Gene expression dataset used for statistical analysis were acquired from the GEO database with the accession code GSE96621. The screening was performed in GEO dataset which contained both the atherosclerotic and non-atherosclerotic ischemic stroke patients. As shown in Figure 1A, the level of miR-155-p was significantly down-expressed in patients with atherosclerosis. To explore the level of miR-155-5p in patients with atherosclerosis, qRT-PCR was applied to determine the level of miR-155-5p in the plasma samples from control patients (n = 67) and patients with atherosclerosis (n = 67). As shown in Figure 1B, the levels of miR-155-5p were remarkably lower in patients with atherosclerosis than that in the control group. To investigate the roles of miR-155-5p in the atherosclerotic process, VSMCs were transfected with miR-155-5p mimic or mi-

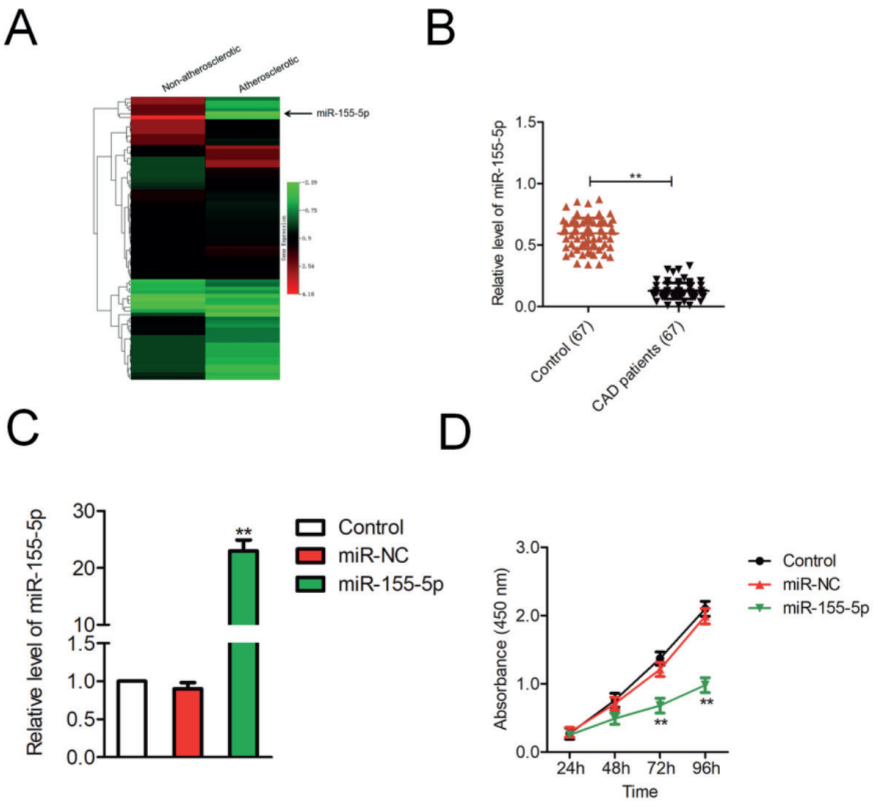

Figure 1. MiR-155-5p was down-regulated in the plasma of patients with atherosclerotic CAD. ***A,*** Microarray analysis of miRNA expression in atherosclerotic and non-atherosclerotic ischemic stroke patients. ***B,*** The levels of miR-155-5p in plasma from controls and patients with atherosclerotic CAD were detected using qRT-PCR assay. ***C,*** The level of miR-155-5p in VSMCs was detected by qRT-PCR. ***D,*** The cell growth was determined using a CCK-8 assay. $^{**}p < 0.01$ compared to control.

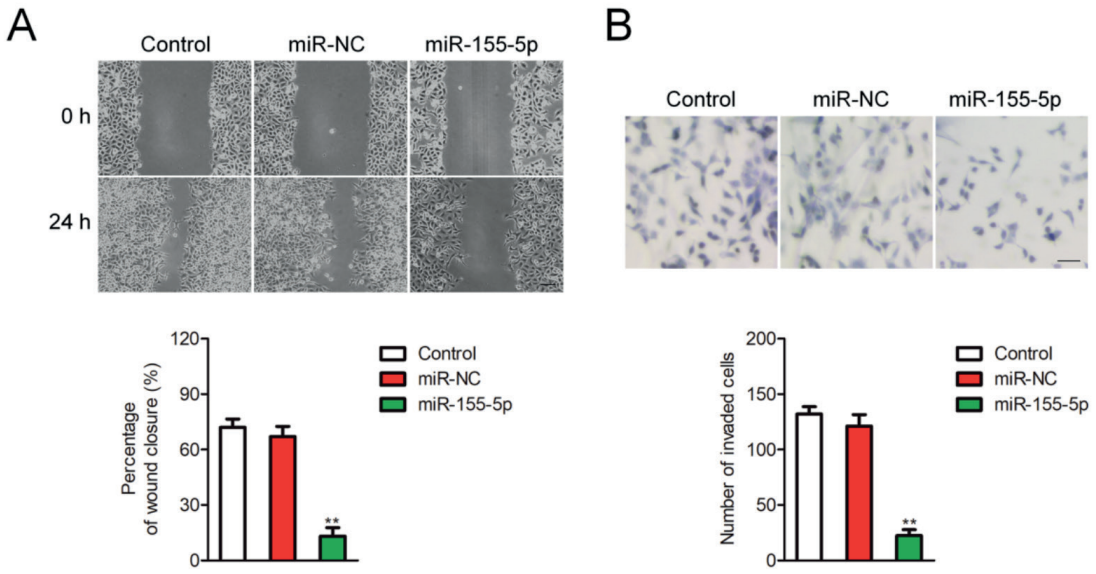

Figure 2. Up-regulation of miR-155-5p inhibits the migration and invasion of VSMCs of VSMCs. ***A,*** VSMCs was transfected with miR-155-5p or miR-NC and the migration of VSMCs was detected using the wound healing assay. B, VSMCs was transfected with miR-155-5p or miR-NC and the invasion of VSMCs was detected using the transwell invasion assay. $^{**}p < 0.01$ compared to control.

mic control (miR-NC). As shown in Figure 1C, the level of miR-155-5p was increased in cell that was transfected with miR-155-5p as demonstrated by qRT-PCR assay. The impact of miR-155-5p on the proliferation in VSMCs was then detected using CCK-8 assay. As shown in Figure 1D, the proliferation of VSMCs was significantly suppressed after cell was transfected with miR-155-5p. All these findings indicated that miR-155-5p was down-regulated in atherosclerosis and up-regulation of miR-155-5p inhibited the proliferation of VSMCs *in vitro*.

Up-regulation of MiR-155-5p Inhibits the Migration and Invasion of VSMCs in vitro

Then, the wound-healing and transwell invasion experiments were conducted to analyze the impacts of miR-155-5p on the migration and invasion of VSMCs *in vitro*. As shown in Figure 2A, the migration of VSMCs cell was significantly inhibited by up-regulation of miR-155-5p. Consistently, the invasion of VSMCs cell was also significantly suppressed by miR-155-5p transfection (Figure 2B). These results indicated that over-regulation of miR-155-5p markedly inhibited the migration and invasion of VSMCs *in vitro*.

Up-regulation of MiR-155-5p Inhibits the Growth, Invasion and Migration of HUVECs in vitro

To investigate the roles of miR-155-5p in the atherosclerotic process, HUVECs were transfected with miR-155-5p or miR-NC. As shown in Figure 3A, the levels of miR-155-5p were increased in cell that was transfected with miR-155-5p as demonstrated by qRT-PCR analysis. Then, we used the CCK-8 assay to determine the effect of miR-155-5p on proliferation in HUVECs *in vitro*. As shown in Figure 3B, the proliferation of HUVECs was remarkably inhibited by miR-155-5p transfection. In addition, the transwell invasion and wound healing experiments were applied to investigate whether miR-155-5p inhibit the invasion and migration of HUVECs. As shown in Figure 3C, the migration of HUVECs that was transfected with miR-155-5p was significantly inhibited. Consistently, the invasion of HUVECs that was transfected with miR-155-5p was also significantly inhibited than cell that was transfected with miR-NC (Figure 3D). These results indicated that over-regulation of miR-155-5p markedly inhibited the migration and invasion of HUVECs *in vitro*.

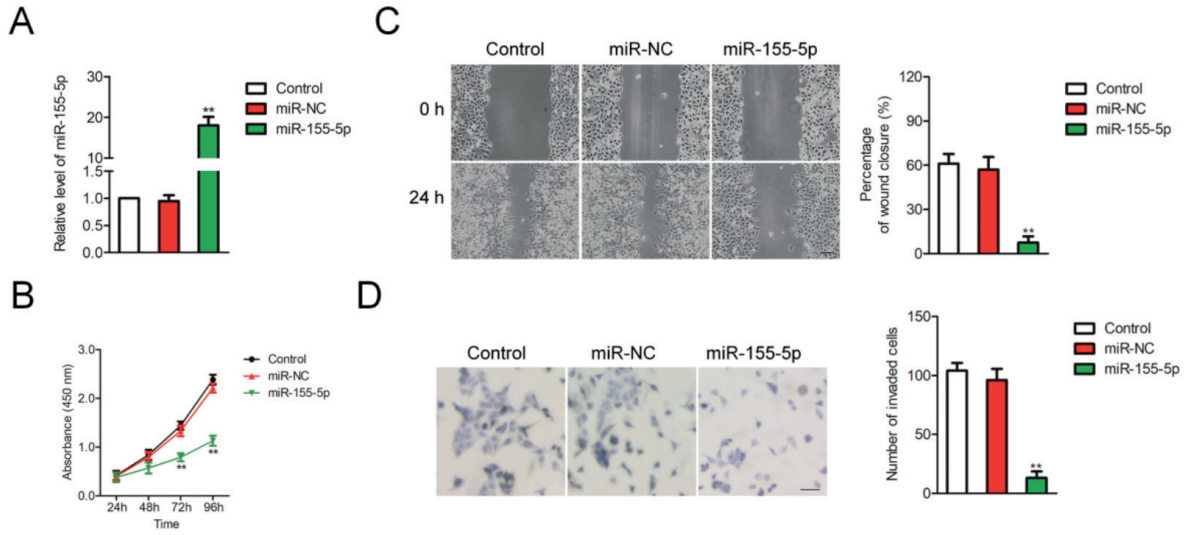

Figure 3. MiR-155-5p inhibits the migration and invasion of HUVECs. ***A,*** HUVECs was transfected with miR-155-5p or miR-NC and the level of miR-155-5p was assessed using qRT-PCR assay. ***B,*** HUVECs was transfected with miR-155-5p or miR-NC and proliferation of HUVECs was detected by CCK-8 assay. ***C,*** HUVECs was transfected with miR-155-5p or miR-NC and the migration ability was analyzed. ***D,*** HUVECs was transfected with miR-155-5p or miR-NC and the invasion of HUVECs was analyzed using the transwell invasion assay. $^{**}p < 0.01$ compared to control.

AKT1 is the Direct Target of MiR-155-5p

Generally, microRNAs regulate its target genes by binding with the 3'-UTR of the target genes. Three bioinformatics analysis tools (TargetScan, miRTarBase and miRDB) were used to predict the target genes of miR-155-5p. The 11 common potential target genes were summarized in Figure 4A-4B. After that, we detected the levels of target genes in cell that was transfected with miR-NC or miR-155-5p. As shown in Figure 4C, the level of AKT1 significantly inhibited in cell that was transfected using miR-155-5p. To verify that AKT1 was the direct target of miR-155-5p, the mutate type (MUT) 3′-UTR of AKT1 or wild type (WT) 3′-UTR of AKT1 that containing the miR-155-5p binding site were constructed into pMIR-REPORT luciferase system. Then, 3′-UTR of AKT1 and miR-155-5p was cotransfected into HEK-293T cell. As shown in Figure 4D, the luciferase activity in HEK-293T cell that was transfected with WT 3′-UTR of AKT1 was significantly inhibited by miR-155-5p, however miR-155-5p transfection did not suppress the luciferase activity in HEK-293T cell that was transfected with MUT 3′-UTR of AKT1. Additional, the expression of AKT1 was significantly down-regulated in VSMCs and HUVECs that were transfected with miR-155-5p (Figure 4E). These results indicated that AKT1 was the target gene of miR-155-5p. Furthermore, the results of ELISA demonstrated that the level of AKT1 in plasma from patients with atherosclerosis was significantly higher than that in the control group (Figure 4F). Collectively, these data strongly suggested that AKT1 was the direct target of miR-155-5p, whose expression was negatively regulated by miR-155-5p.

MiR-155-5p Inhibits the Proliferation, Invasion and Migration of VSMCs and HUVECs by Binding AKT1

Since AKT1 is the direct target of miR-155-5p, we further verified whether the effect of miR-155-5p on the proliferation, migration and invasion of VSMCs and HUVECs depended on AKT1. Hence, VSMCs and HUVECs were cotransfected with miR-155-5p and pcDNA3.1-AKT1 plasmid (Figure 5A). Then, the proliferation assay showed that the effect of miR-155-5p on the proliferation of VSMCs and HUVECs was rescued by over-regulation of AKT1 (Figure 5B). In addition, the wound healing and transwell invasion assay indicated that the inhibitory impact of miR-155-5p on VSMCs and HUVECs migration and invasion was neutralized by over-expression of AKT1 (Figure 5C-5D). In conclusion, these findings indicated that miR-101-5p inhibited the proliferation and aggressive phenotype of VSMCs and HUVECs by targeting AKT1.

Discussion

Atherosclerosis, which causes many deaths through myocardial infarction, peripheral vascular disease, ischemic stroke and heart attack, is a major medical and socioeconomic problem[29,30]. Substantial investigations suggest that the aberrant proliferation, migration and invasion of HUVECs and VSMCs are the essential regulators of atherosclerosis[31-33]. Hence, de-

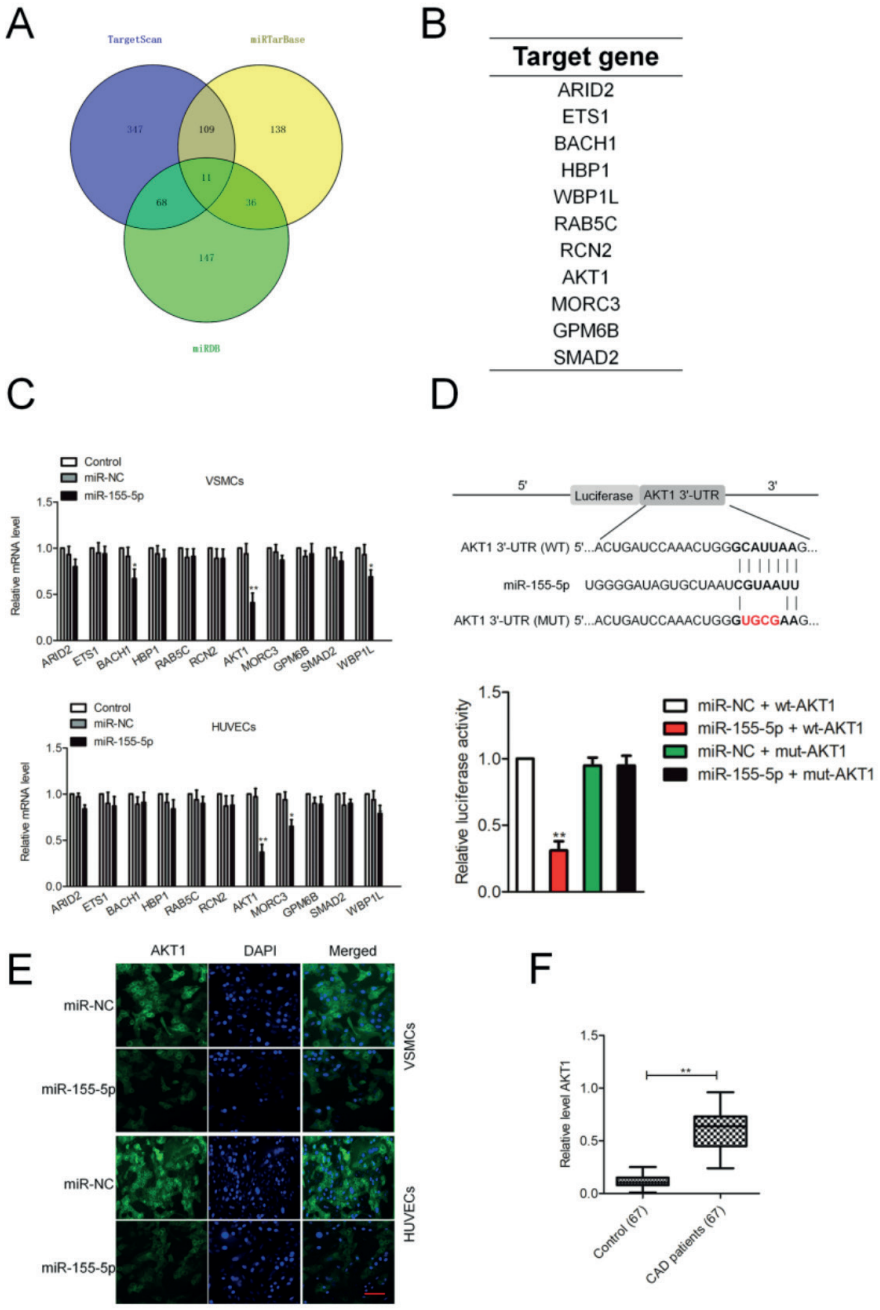

Figure 4. The direct target gene of miR-155-5p is AKT1. ***A-B,*** Venn graph represented the number of common target genes that were determined by three bioinformatics analysis. ***C,*** VSMCs or HUVECs was transfected with miR-NC or miR-155-5p and the levels of common target genes were detected using qRT-PCR assay. $^{**}p < 0.01$, compared to control. ***D,*** The potential binding site between AKT1 and miR-155-5p was identified (upper panel). The relative luciferase activity in HEK293 cells was detected using luciferase reporter gene assay (lower panel). $^{**}p < 0.01$, compared to miR-NC + WT-AKT1. ***E,*** Cell was transfected with miR-NC or miR-155-5p, and the level of AKT1 was detected by immunofluorescence staining assay. ***F,*** The levels of AKT1 in the plasma from control or patients with CAD were detected by ELISA assay. $^{**}p < 0.01$, compared to control.

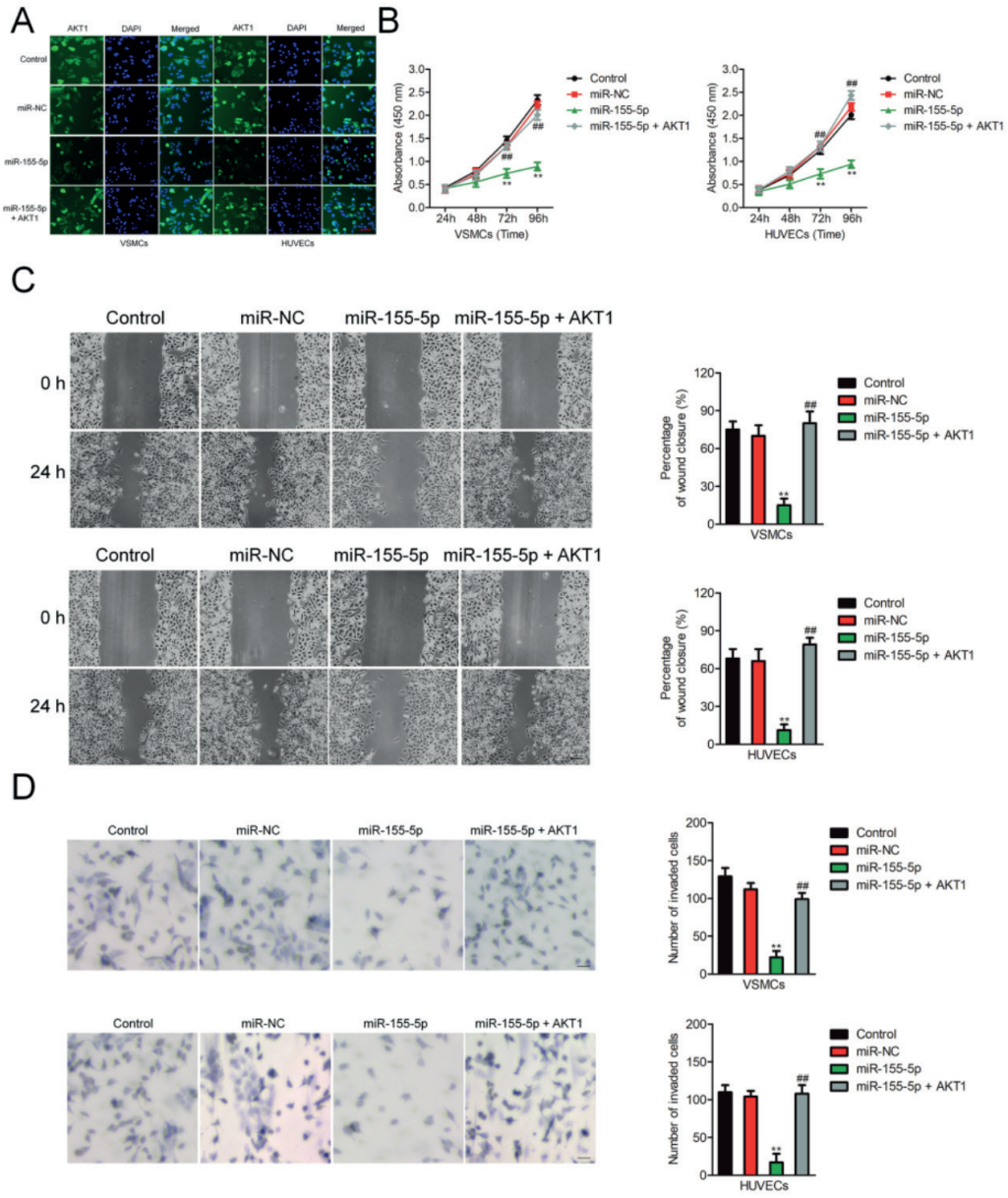

Figure 5. MiR-155-5p inhibits the migration, proliferation and invasion of VSMCs and HUVECs via regulating AKT1. ***A,*** VSMCs and HUVECs was transfected with miR-155-5p or cotransfected with miR-55-5p and pcDNA3.1-AKT1 plasmid. The expression of AKT1 was measured by immunofluorescence staining assay. ***B,*** VSMCs and HUVECs were transfected with miR-155-5p or cotransfected with miR-155-5p and pcDNA3.1-AKT1 plasmid. The proliferation assay was conducted. ***C,*** VSMCs and HUVECs were transfected with miR-101-5p alone or cotransfected with miR-101-5p and pcDNA3.1-AKT1 plasmid. ***D,*** The migration of VSMCs and HUVECs was measured by wound healing assay. ***E,*** The invasion of VSMCs and HUVECs was detected using transwell invasion assay. $^{**}p < 0.01$, compared to miR-NC. $^{\#\#}p < 0.01$, compared to cell cotransfected with miR-155-5p and pcDNA3.1-AKT1.

termining the molecular mechanisms by which HUVECs and VSMCs contribute to atherosclerosis is critical for developing new diagnostic and therapeutic strategies for atherosclerosis. Previous studies have indicated that miR-155-5p is closely related to the development of various types of cancers34-36. Surprisingly, down-regulation of miR-155-5p is related with the ischemic stroke37. But, the potential effect of miR-155-5p and its potential mechanisms in the growth, migration and invasion of VSMCs and HUVECs has not been investigated in atherosclerosis. In

the current paper, we found that miR-155-5p was down-regulated in the plasma samples of patients with atherosclerotic CAD. We further studied the precise role of miR-155-5p in atherosclerosis. *In vitro* functional experiments indicated that up-regulation of miR-155-5p inhibited the proliferation, migration and invasion of VSMCs and HUVECs. MiRNAs have been confirmed by binding to the target gene 3'-UTR to regulate the expressions of protein-encoding genes. To further explore the molecular mechanisms underlying the actions of miR-155-5p in atherosclerosis, we identified the potential functional target gene of miR-155-5p using online bioinformatics analysis tools and luciferase reporter assay. The results indicated that the 3'-UTR of AKT1 contained the binding sites between miR-155-5p. Previous investigations have indicated that AKT1 plays an important role in many physiopathological processes[38,39]. It has been found that the phosphoinositide-3-Kinase-AKT Serine/Threonine Kinase 1 (PI3K-AKT) signaling axis participates into the regulation of cellular metabolism, gene expression, cell survival, and migration in a variety of cells. In the vascular wall, AKT plays an important role in the proliferation and migration of endothelial cell as well as the regulation of the vascular permeability and angiogenesis[40,41]. Recent investigations using AKT knockout mice also indicate that AKT1 is a major AKT isoform expressed in endothelial cell[42]. AKT1 deletion reduced endothelial cell migration, endothelial nitric oxide synthase (eNOS) phosphorylation, ischemia-induced angiogenesis, nitric oxide (NO) secretion, and vascular endothelial growth factor (VEGF) damage[43].

A growing body of investigations indicate that diverse miRNAs regulate the expression of AKT1 in many diseases44. Nevertheless, whether miR-155-5p directly regulated the expressions of AKT1 in VSMCs and HUVECs remained unclear. The luciferase reporter assay proved that AKT1 was the direct target gene of miR-155-5p. Over-expression of miR-155-5p significantly decreased the mRNA and protein levels of AKT1. We also revealed that the levels of AKT1 in patients with atherosclerotic CAD were higher than in the control group. Notably, co-transfection of miR-155-5p and AKT1 remarkably increased the protein expression of AKT1 and rescued the inhibitory effect of miR-155-5p on the proliferation, migration and invasion of VSMCs and HUVECs.

Conclusions

We demonstrated that miR-155-5p was under-regulated in patients with atherosclerotic CAD. Over-regulation of miR-155-5p suppressed the proliferation, migration and invasion of VSMCs and HUVECs thorough targeting AKT1. The present study provided the evidence about the involvement of miR-155-5p in the pathogenesis of atherosclerosis.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Katakami N. Mechanism of development of atherosclerosis and cardiovascular disease in diabetes mellitus. J Atheroscler Thromb 2018; 25: 27-39.
2) Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med 2011; 17: 1410-1422.
3) Pan JX. LncRNA H19 promotes atherosclerosis by regulating MAPK and NF-kB signaling pathway. Eur Rev Med Pharmacol Sci 2017; 21: 322-328.
4) Stylianou IM, Bauer RC, Reilly MP, Rader DJ. Genetic basis of atherosclerosis: insights from mice and humans. Circ Res 2012; 110: 337-355.
5) Escarcega RO, Lipinski MJ, Garcia-Carrasco M, Mendoza-Pinto C, Galvez-Romero JL, Cervera R. Inflammation and atherosclerosis: cardiovascular evaluation in patients with autoimmune diseases. Autoimmun Rev 2018; 17: 703-708.
6) Yan HY, Bu SZ, Zhou WB, Mai YF. TUG1 promotes diabetic atherosclerosis by regulating proliferation of endothelial cells via Wnt pathway. Eur Rev Med Pharmacol Sci 2018; 22: 6922-6929.
7) Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, Swiatlowska P, Newman AA, Greene ES, Straub AC, Isakson B, Randolph GJ, Owens GK. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. Nat Med 2015; 21: 628-637.
8) Bian F, Yang X, Zhou F, Wu PH, Xing S, Xu G, Li W, Chi J, Ouyang C, Zhang Y, Xiong B, Li Y, Zheng T, Wu D, Chen X, Jin S. C-reactive protein promotes atherosclerosis by increasing LDL transcytosis across endothelial cells. Br J Pharmacol 2014; 171: 2671-2684.
9) Wang L, Qiu XM, Hao Q, Li DJ. Anti-inflammatory effects of a Chinese herbal medicine in atherosclerosis via estrogen receptor beta mediating nitric oxide production and NF-kappaB suppression in endothelial cells. Cell Death Dis 2013; 4: e551.
10) Pavlides S, Gutierrez-Pajares JL, Iturrieta J, Lisanti MP, Frank PG. Endothelial caveolin-1 plays a major

role in the development of atherosclerosis. Cell Tissue Res 2014; 356: 147-157.

11) Yang LL, Liu JQ, Bai XZ, Fan L, Han F, Jia WB, Su LL, Shi JH, Tang CW, Hu DH. Acute downregulation of miR-155 at wound sites leads to a reduced fibrosis through attenuating inflammatory response. Biochem Biophys Res Commun 2014; 453: 153-159.

12) Brennan E, Wang B, McClelland A, Mohan M, Marai M, Beuscart O, Derouiche S, Gray S, Pickering R, Tikellis C, de Gaetano M, Barry M, Belton O, Ali-Shah ST, Guiry P, Jandeleit-Dahm KAM, Cooper ME, Godson C, Kantharidis P. Protective effect of let-7 miRNA family in regulating inflammation in diabetes-associated atherosclerosis. Diabetes 2017; 66: 2266-2277.

13) Vacca M, Di Eusanio M, Cariello M, Graziano G, D'Amore S, Petridis FD, D'Orazio A, Salvatore L, Tamburro A, Folesani G, Rutigliano D, Pellegrini F, Sabba C, Palasciano G, Di Bartolomeo R, Moschetta A. Integrative miRNA and whole-genome analyses of epicardial adipose tissue in patients with coronary atherosclerosis. Cardiovasc Res 2016; 109: 228-239.

14) Wu XD, Zeng K, Liu WL, Gao YG, Gong CS, Zhang CX, Chen YQ. Effect of aerobic exercise on miRNA-TLR4 signaling in atherosclerosis. Int J Sports Med 2014; 35: 344-350.

15) Toba H, Cortez D, Lindsey ML, Chilton RJ. Applications of miRNA technology for atherosclerosis. Curr Atheroscler Rep 2014; 16: 386.

16) Liao CG, Kong LM, Zhou P, Yang XL, Huang JG, Zhang HL, Lu N. miR-10b is overexpressed in hepatocellular carcinoma and promotes cell proliferation, migration and invasion through RhoC, uPAR and MMPs. J Transl Med 2014; 12: 234.

17) Qu R, Hao S, Jin X, Shi G, Yu Q, Tong X, Guo D. MicroRNA-374b reduces the proliferation and invasion of colon cancer cells by regulation of LRH-1/Wnt signaling. Gene 2018; 642: 354-361.

18) Li M, Liu Q, Lei J, Wang X, Chen X, Ding Y. MiR-362-3p inhibits the proliferation and migration of vascular smooth muscle cells in atherosclerosis by targeting ADAMTS1. Biochem Biophys Res Commun 2017; 493: 270-276.

19) Xu Z, Han Y, Liu J, Jiang F, Hu H, Wang Y, Liu Q, Gong Y, Li X. MiR-135b-5p and MiR-499a-3p promote cell proliferation and migration in atherosclerosis by directly targeting MEF2C. Sci Rep 2015; 5: 12276.

20) Zhao W, Zhao SP, Zhao YH. MicroRNA-143/-145 in cardiovascular diseases. Biomed Res Int 2015; 2015: 531740.

21) Guo X, Li D, Chen M, Chen L, Zhang B, Wu T, Guo R. miRNA-145 inhibits VSMC proliferation by targeting CD40. Sci Rep 2016; 6: 35302.

22) Wu R, Tang S, Wang M, Xu X, Yao C, Wang S. MicroRNA-497 induces apoptosis and suppresses proliferation via the Bcl-2/Bax-caspase9-caspase3 pathway and cyclin D2 protein in HUVECs. PLoS One 2016; 11: e0167052.

23) Shi Y, Fu X, Cao Q, Mao Z, Chen Y, Sun Y, Liu Z, Zhang Q. Overexpression of miR-155-5p inhibits the proliferation and migration of IL-13-induced human bronchial smooth muscle cells by suppressing TGF-beta-activated kinase 1/MAP3K7-binding protein 2. Allergy Asthma Immunol Res 2018; 10: 260-267.

24) Mogi M, Walsh K, Iwai M, Horiuchi M. Akt-FOXO3a signaling affects human endothelial progenitor cell differentiation. Hypertens Res 2008; 31: 153-159.

25) Tsuchiya K, Westerterp M, Murphy AJ, Subramanian V, Ferrante AW, Jr., Tall AR, Accili D. Expanded granulocyte/monocyte compartment in myeloid-specific triple FoxO knockout increases oxidative stress and accelerates atherosclerosis in mice. Circ Res 2013; 112: 992-1003.

26) Rotllan N, Wanschel AC, Fernandez-Hernando A, Salerno AG, Offermanns S, Sessa WC, Fernandez-Hernando C. Genetic evidence supports a major role for Akt1 in VSMCs during atherogenesis. Circ Res 2015; 116: 1744-1752.

27) Fernandez-Hernando C, Jozsef L, Jenkins D, Di Lorenzo A, Sessa WC. Absence of Akt1 reduces vascular smooth muscle cell migration and survival and induces features of plaque vulnerability and cardiac dysfunction during atherosclerosis. Arterioscler Thromb Vasc Biol 2009; 29: 2033-2040.

28) Fernandez-Hernando C, Ackah E, Yu J, Suarez Y, Murata T, Iwakiri Y, Prendergast J, Miao RQ, Birnbaum MJ, Sessa WC. Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. Cell Metab 2007; 6: 446-457.

29) Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473: 317-325.

30) Wilck N, Ludwig A. Targeting the ubiquitin-proteasome system in atherosclerosis: status quo, challenges, and perspectives. Antioxid Redox Signal 2014; 21: 2344-2363.

31) Zhao Y, Zhang C, Wei X, Li P, Cui Y, Qin Y, Wei X, Jin M, Kohama K, Gao Y. Heat shock protein 60 stimulates the migration of vascular smooth muscle cells via Toll-like receptor 4 and ERK MAPK activation. Sci Rep 2015; 5: 15352.

32) Yao X, Yan C, Zhang L, Li Y, Wan Q. LncRNA ENST00113 promotes proliferation, survival, and migration by activating PI3K/Akt/mTOR signaling pathway in atherosclerosis. Medicine (Baltimore) 2018; 97: e0473.

33) Zhu N, Zhang D, Chen S, Liu X, Lin L, Huang X, Guo Z, Liu J, Wang Y, Yuan W, Qin Y. Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. Atherosclerosis 2011; 215: 286-293.

34) Bhattacharya S, Chalk AM, Ng AJ, Martin TJ, Zannettino AC, Purton LE, Lu J, Baker EK, Walkley CR. Increased miR-155-5p and reduced miR-148a-3p contribute to the suppression of osteosarcoma cell death. Oncogene 2016; 35: 5282-5294.

35) Wang M, Yang F, Qiu R, Zhu M, Zhang H, Xu W, Shen B, Zhu W. The role of mmu-miR-155-5p-NF-kappaB signaling in the education of bone marrow-derived mesenchymal stem cells by gastric cancer cells. Cancer Med 2018; 7: 856-868.

36) Wang Q, Li C, Zhu Z, Teng Y, Che X, Wang Y, Ma Y, Wang Y, Zheng H, Liu Y, Qu X. miR-155-5p antagonizes the apoptotic effect of bufalin in triple-negative breast cancer cells. Anticancer Drugs 2016; 27: 9-16.

37) Greco R, Demartini C, Zanaboni AM, Blandini F, Amantea D, Tassorelli C. Endothelial nitric oxide synthase inhibition triggers inflammatory responses in the brain of male rats exposed to ischemia-reperfusion injury. J Neurosci Res 2018; 96: 151-159.

38) Hers I, Vincent EE, Tavare JM. Akt signalling in health and disease. Cell Signal 2011; 23: 1515-1527.

39) Abeyrathna P, Su Y. The critical role of Akt in cardiovascular function. Vascul Pharmacol 2015; 74: 38-48.

40) Haslinger P, Haider S, Sonderegger S, Otten JV, Pollheimer J, Whitley G, Knofler M. AKT isoforms 1 and 3 regulate basal and epidermal growth factor-stimulated SGHPL-5 trophoblast cell migration in humans. Biol Reprod 2013; 88: 54.

41) Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002; 14: 381-395.

42) Lee MY, Gamez-Mendez A, Zhang J, Zhuang Z, Vinyard DJ, Kraehling J, Velazquez H, Brudvig GW, Kyriakides TR, Simons M, Sessa WC. Endothelial cell autonomous role of Akt1: regulation of vascular tone and ischemia-induced arteriogenesis. Arterioscler Thromb Vasc Biol 2018; 38: 870-879.

43) Symons JD, McMillin SL, Riehle C, Tanner J, Palionyte M, Hillas E, Jones D, Cooksey RC, Birnbaum MJ, McClain DA, Zhang QJ, Gale D, Wilson LJ, Abel ED. Contribution of insulin and Akt1 signaling to endothelial nitric oxide synthase in the regulation of endothelial function and blood pressure. Circ Res 2009; 104: 1085-1094.

44) Rajan KS, Velmurugan G, Pandi G, Ramasamy S. miRNA and piRNA mediated Akt pathway in heart: antisense expands to survive. Int J Biochem Cell Biol 2014; 55: 153-156.