European Review for Medical and Pharmacological Sciences 2019; 23: 8540-8550

Downregulation of linc00961 contributes to promote proliferation and inhibit apoptosis of vascular smooth muscle cell by sponging miR-367 in patients with coronary heart disease

C.-T. WU, S. LIU, M. TANG

Department of Cardiac Surgery, Second Hospital of Hebei Medical University, Shi Jiazhuang, China

Abstract. – OBJECTIVE: Atherosclerosis is one of the most important risk factors for coronary heart disease (CHD), and growing evidence has shown that long non-coding RNAs (lncRNAs) can serve as prospective markers for atherosclerosis. In this study, we mainly focused on the potential roles of linc00961 in CHD patients.

PATIENTS AND METHODS: qRT-PCR was used to detect the expressions of linc00961 and miR-367 in CHD patients and ApoE-/-mice, and the correlations were analyzed. Then, HA-VAMC was respectively treated with 5 inflammatory factors and hypoxia conditions to explore the factors that affect linc00961 levels. Furthermore, the linc00961 overexpression lentivirus (LV-linc00961) and linc00961 downregulation lentivirus (LV-sh linc00961) were purchased and transfected into human vascular smooth muscle cells (VSMCs). CCK8 assay was carried out to measure the cell proliferation of VSMC, and the levels of Cyclin D1, Bcl-2, Bax, and cleaved caspase-3 were detected by RT-PCR and Western blot. Moreover, the Luciferase assay was performed to explore the binding site of linc00961 and miR-367. Finally, the miR-367 inhibitor was transfected into LV-sh linc00961 VSMCs to confirm the linc00961 functions via miR-367.

RESULTS: We found that linc00961 was significantly decreased in patients with CHD and ApoE-/-mice. Additionally, linc00961 was reduced in VSMCs at the conditions of hypoxia and C-reactive protein (CRP). Most importantly, the overexpression of linc00961 significantly inhibited the VSMCs proliferation, repressed the levels of Cyclin D1 and Bcl-2, and increased the levels of Bax and cleaved caspase-3. However, the downregulation of linc00961 promoted VSMCs proliferation, increased the levels of Cyclin D1 and Bcl-2, and repressed the levels of Bax and cleaved caspase-3. We also found that miR-367 was downregulated following the upregulation of linc00961, while it was upregulated following the downregulation of linc00961. The Luciferase gene reporter assay indicated that linc00961 could directly bind with miR-367 in VSMCs. Finally, we found that linc00961 could inhibit proliferation and promote apoptosis of VSMCs via binding with miR-367.

CONCLUSIONS: According to the results, our study revealed that linc00961 was significantly decreased in patients with CHD and ApoE-/-mice. Furthermore, our findings firstly uncovered that linc00961 was reduced by hypoxia and CRP in VSMCs. The downregulation of linc00961 contributed to promote proliferation and inhibit apoptosis of VSMCs by sponging miR-367 in CHD patients, which might provide a potential target for treating atherosclerosis.

Key Words:
Linc00961, MiR-367, Proliferation, Atherosclerosis, Coronary heart disease.

Introduction

Atherosclerosis is a disease of large elastic and muscular arteries, which is the most dangerous factor and responsible for coronary heart disease (CHD)[1-3]. It is reported that more than 20 million people die every year because of CHD, making it one of the leading causes of mortality in the world[3-5]. The activation of the inflammatory cells, such as lymphocytes and monocyte-derived macrophages leads to the secretion of inflammatory cytokines, such as IL-2, IL-6, IL-8, IL-15, IL-18, IFN-γ, and TNF-α, which will promote the development of atherosclerosis and CHD[6-9]. Moreover, the proliferation of vascular smooth muscle cells (VSMCs) and the formation of neo-intima dominate the development of atherosclerosis[10-13].

Long non-coding RNAs (lncRNAs) are a sort of RNAs that are more than 200 nucleotides (nt)

Corresponding Author: Chun Tao Wu, MD; e-mail: drchuntaowu@hotmail.com

but have no protein-coding capacity. They have been found to participate in many biological processes in various diseases[14-16]. LncRNAs have been widely found to be aberrantly expressed in many cancers[17-20]. Recently, it has been reported that lncRNAs can be involved in the regulation and development of cardiovascular diseases, such as atherosclerosis and CHD[21,22]. For example, lncRNA TUG1 could promote the proliferation of VSMCs and atherosclerosis by regulating miRNA-21/PTEN axis[23]; lncRNA MIAT could inhibit efferocytosis in advanced atherosclerosis by sponging miR-149-5p[24]. Long non-coding RNA 00961 (Linc00961) is located in chromosome 9, which is 1,546 nucleotides in length[25]. It has been reported to be a tumor suppressor in multiple types of cancers, including skin melanoma[26], oral squamous cell carcinoma[27], renal cell carcinoma[28], etc. However, the expression and biological function of linc00961 in atherosclerosis and CHD are less understood.

MiRNAs have been observed to be involved in many biological processes of various diseases, and miR-367 was found to participate in the carcinogenesis and progression in multiple types of cancers[29,30]. For example, miR-367 could enhance the proliferation and invasion of cutaneous malignant melanoma[31]; miR-367 participated in regulating cell proliferation and metastasis in clear-cell renal cell carcinoma[32]. However, the role of miR-367 in atherosclerosis and CHD is unclear.

In this study, we aimed at investigating the expression and function of linc00961 in CHD. We observed a significant decrease of linc00961 expression in CHD patients and ApoE-/-mice, and the downregulation of linc00961 attenuated to the development of CHD. Furthermore, we also detected that miR-367 expression was increased. Therefore, we speculated that linc00961 could play an important role in the development of CHD by regulating miR-367.

Patients and Methods

Patients and Animals

20 serum samples from CHD patients and 20 serum samples from healthy volunteers were collected in our hospital from July 2016 to September 2017. All samples were frozen in liquid nitrogen at -80°C until use. 20 ApoE-/-mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd, (Beijing, China). These ApoE-/-mice received a high-fat diet, which was kept in the standard specific pathogen-free (SPF) environment with 12 hours light and 12 hours dark at the temperature of 20-25°C. These mice were rapidly euthanized in cervical paralysis after 16 weeks, and the atherosclerotic plaque and adjacent tissue were frozen in liquid nitrogen at -80°C until use. Our study was approved by the Faculty of Medicine's Ethics Committee of our hospital. All animal experiments were performed in accordance with institutional guidelines. The human study was performed in accordance with the principles of the Declaration of Helsinki and was approved by the Ethical Committee. Informed consent was obtained from each patient.

Cell Culture

Human vascular smooth muscle cell line HA-VSMC was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), the antibiotics penicillin (100 U/ml), and streptomycin (100 μg/ml), and cells were cultured in an appropriate incubator with 37°C and 5% CO_2.

Construction of Lentivirus and Cell Transfection

The full length of human linc00961 cDNA was synthesized and subcloned into a lentivirus (Shanghai GenePharma Co, Ltd, Shanghai, China), resulting in linc00961 overexpression, named LV-linc00961. And the short hairpin linc00961 (sh linc00961) cDNA was synthesized and cloned into the lentivirus, resulting in linc00961 downregulation, which named LV-sh linc00961. LV-linc00961 and LV-sh linc00961 and their negative control (NC) were respectively infected into VSMCs for 24 hours according to the manufacturer's protocol; then, the transfection efficiency was observed under a fluorescent inverted microscope. The stable VSMC cell lines with linc00961 overexpression or linc00961 downregulation were constructed after 1 to 2 weeks. The cells were prepared and harvested for further study. VSMCs were seeded in 6-well plates (1×10^6/well) until reaching 70%; before transfection, the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), serum-free DMEM and miR-367 NC or miR-367 mimic were mixed and incubated for 30 mins, which were then added into VSMCs with complete medium containing

15% FBS. At the indicated time point after transfection, the cells were harvested for further study.

CCK8 Assay

VSMCs were respectively transfected with LV-NC, LV-linc00961, LV-sh linc00961, or co-transfected with LV-sh linc00961 and miR-367 inhibitor for 48 h. After that, the target cells were seeded in 96-well plates (2×10^3/well) and cultured in DMEM/F12 medium at 37°C and 5% CO_2 for 1 d, 3 d, and 7 d. Three replicate wells were set in each group. 10 ul Cell Counting Kit-8 (CCK-8, Dojundo, Kumamoto, Japan) was added into 100 ul of DMEM medium in each well, which was co-cultured at darkness for 2 hours at 37°C. Next, the proliferation of VSMCs was measured by CCK8 assay. The absorbance (OD) value of each well was measured at 450 nm with microplate reader (Thermo Fisher, Waltham, MA, USA). The data was collected for 1 d, 3 d, and 7 d. The whole experiment was repeated three times.

RNA Extraction and quantitative Real Time-PCR

The total RNA of serum was extracted by using TRIzol LS (Invitrogen, Carlsbad, CA, USA), and the total RNA of mice tissues and cells was extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription was performed by using the PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the protocol. PCR primers were synthesized by Gene Pharma (Shanghai Gene Pharma, Shanghai, China), and the sequences were listed in Table I. mRNA expressions were detected by SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan). The mRNA expressions of linc00961, Cyclin D1, Bcl-2, Bax, and cleaved caspase-3 were normalized to β-actin, and miR-367 was normalized to U6 and $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expressions.

Protein Extraction and Western Blot

The cells were harvested at a density of 80%, and the total protein was extracted by using a RIPA lysis buffer (Biyuntian, Shanghai, China). The protein concentration was measured with the BCA kit (Sigma-Aldrich, St. Louis, MO, USA) according to its protocol. 50 μg samples were added to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, (SDS-PAGE) and then, the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were blocked in the 5% non-fat milk at room temperature for 1 h. They were washed with Tris-Buffered Saline and Tween 20 (TBST; Boster, China) for three times and then the membranes were respectively incubated with primary antibodies (anti-Cyclin D1, 1:200, 33 kDa; anti-caspase-3, 1:500, 32 kDa; anti-Bax, 1:1000, 20 kDa; anti-Bcl-2, 1:1000, 26 kDa; β-actin, 1:1000, 45 kDa) overnight at 4°C. All primary antibodies were bought from Cell

Table I. Sequences of primers for RT-PCR

Genes	Primer sequences
Linc00961	Forward: 5'- GCAGAATGCCATGGTTTCCC -3' Forward: 5'-CTGTTCTGGATGGGAGCGAA-3' Reverse: 5'-ACAGTCACCACGAACAGCAC-3'
miR-367	Forward: 5'-TTCTCCGAACTTTGCACGTTT-3' Reverse: 5'-ACGTGACACGTTCGGAGAATT-3'
Cyclin D1	Forward: 5'-AGCTGTGCATCTACACCGAC-3' Reverse: 5'-TGTGAGGCGGTAGTAGGACA-3'
Bax	Forward: 5'-GCGACTGATGTCCCTGTCTC-3' Reverse: 5'-AAAGATGGTCACGGTCTGCC-3'
Bcl-2	Forward: 5'-CTCCCACAGACTCTGTAAG-3' Reverse: 5'-GCATTACCTGGGGCTGTAATT-3'
Caspase3	Forward: 5'-ATTTGGAACCAAAGATCATACA-3' Reverse: 5'-CTGAGGTTTGCTGCATCGAC-3'
β-actin	Forward: 5'-CCAAGGCCAACCGCGAGAAGAT-3' Forward: 5'-AGGGTACATGGTGGTGCCGCCA-3'
U6	Forward: 5'-CGCTTCGGCAGCACATATACT-3' Forward: 5'-CGCTTCACGAATTTGCGTGTC-3'

Signaling Technology (CST, Danvers, MA, USA). Then, they were subsequently incubated with matched the secondary antibody (1:5000) for 1 h. The protein bands were detected by Pierce ECL Western blot substrate (Thermo Fisher Scientific, Shanghai, China) with enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Shanghai, China) and analyzed by using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Luciferase Assay

The potential 3'UTR binding sequence was predicted by starBase v2.0 database, the sequences of linc00961-wt and mutant sequence linc00961-mut were synthesized and cloned into pmiR-GLO (Promega, Madison, WI, USA). VSMCs were seeded in 48-well plates for 24 h. Then, miR-367 mimic and miR-NC were co-transfected into VSMCs with pGL3-WT/mut-linc00961 for 24 h. The plasmids (200 ng) were mixed with Lipofectamine 2000 and DMEM medium for 20 mins, then, the mixtures were added into VSMCs for 24 h. After transfection for 24 h, the cells were lysed, and firefly and Renilla Luciferase activities were measured by using Dual-Luciferase reporter assay (Promega, Madison, WI, USA). Data were normalized against the activity of the Renilla Luciferase gene, and the ratio of these two revealed the relative activity of Luciferase.

Statistical Analysis

The data were expressed as the mean±SD, which was analyzed by SPSS 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). The significance between the groups was analyzed by the Student's *t*-test or One-way ANOVA, and multiple comparisons between the groups were performed by the SNK method after ANOVA analysis. If p-value<0.05, it was considered statistically significant.

Results

Linc00961 Was Reduced in Patients with CHD and ApoE-/-Mice

To explore the function of linc00961 in atherosclerosis and CHD, we first used qRT-PCR to detect the expression of linc00961 in the serum samples from 20 patients, compared with 20 healthy volunteers. The results showed that linc00961 was significantly repressed in patients with CHD (Figure 1A) (p<0.05). Furthermore, we also detected the expression of linc00961 in aortic atherosclerotic plaques of ApoE-/-mice, which was a widely used animal model of atherosclerosis. As a result, it was shown that linc00961 was substantially lower in the aortic plaques of ApoE-/-mice, compared with the wild type control mice (Figure 1B) (p<0.05). These results indicated that linc00961 was downregulated in patients with CHD and ApoE-/-mice, which might play some roles in the procession of atherosclerosis, but the detailed mechanism remained unknown.

Linc00961 Was Reduced by Hypoxia or CRP in VSMCs

The endothelial cell injury plays an important role in the development of atherosclerosis

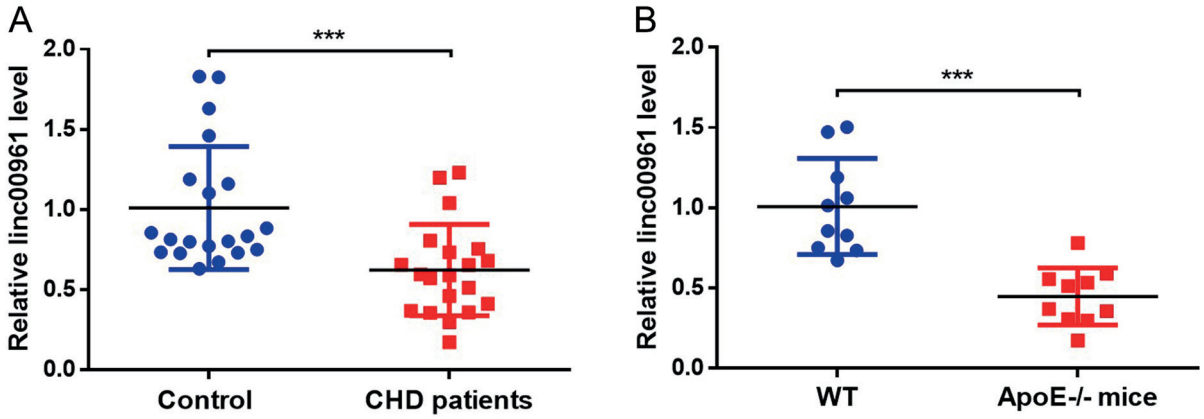

Figure 1. Linc00961 was reduced in patients with CHD and ApoE-/-mice. **A,** The mRNA levels of linc00961 in serum samples of CHD patients (n=20) and healthy volunteer (n=20) were detected by RT-PCR. **B,** The mRNA levels of linc00961 in aortic atherosclerotic plaques of ApoE-/-mice (n=10) and wild type mice (n=10) were detected by RT-PCR. ***p<0.001.

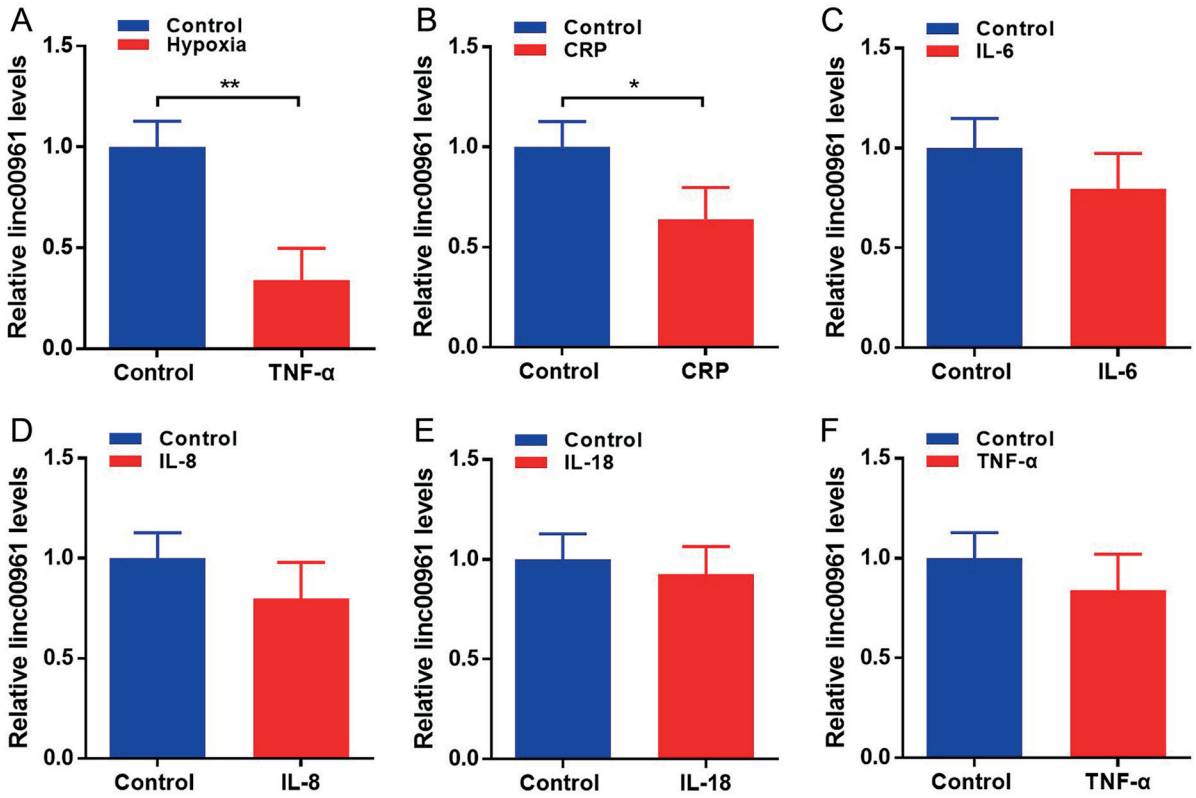

Figure 2. Linc00961 was reduced by hypoxia or CRP in VSMCs. **A,** The mRNA level of linc00961 in VSMCs was detected by RT-PCR in hypoxia condition. **B-F,** The mRNA levels of linc00961 in VSMCs were detected by RT-PCR after the treatment of inflammatory factors, such as CRP, IL-6, IL-8, IL-18, TNF-α. Data are shown as mean ± SD based on at least three independent experiments. *p<0.05, **p<0.01.

and CHD. In order to evaluate the factors that affect the linc00961 expression, human vascular smooth muscle cell line HA-VAMC was respectively treated with inflammatory factors (CRP, IL-6, IL-8, IL-18, TNF-α) and hypoxia condition (4% O_2). After being treated for 24 h, the linc00961 expressions were detected by using RT-PCR. The results revealed that the linc00961 level was repressed in hypoxia condition (Figure 2A) (p<0.05). Furthermore, the HA-VSMC was inhibited in C-reactive protein (CRP) condition (20 ng/ml) (Figure 2B) (p<0.05), while no significant difference has been found in the conditions of IL-6, IL-8, IL-18, TNF-α (Figure 2C-2F) (p<0.05). These findings indicated that linc00961 was reduced in VSMCs induced with hypoxia and CRP, which might provide a valuable research orientation for further investigation.

Upregulation of Linc00961 Inhibited Proliferation and Promoted Apoptosis of VSMCs

To further explore the functions and roles of linc00961 in atherosclerosis, the LV- linc00961 was constructed, which resulted in linc00961 overexpression. After LV- linc00961 infection into VSMCs, the linc00961 level was significantly increased (Figure 3A) (p<0.001), indicating that linc00961 was successfully infected into VSMCs. Then, CCK8 assay was performed to evaluate the proliferation of VSMCs; the results showed that the overexpression of linc00961 significantly inhibited the VSMCs proliferation after 3 d and 7 d, compared with the control group (Figure 3B) (p<0.05). Furthermore, we detected Cyclin D1 expression, which was a critical factor to promote cell proliferation, and we also detected some apoptotic gene expressions, such as Bcl-2, Bax,

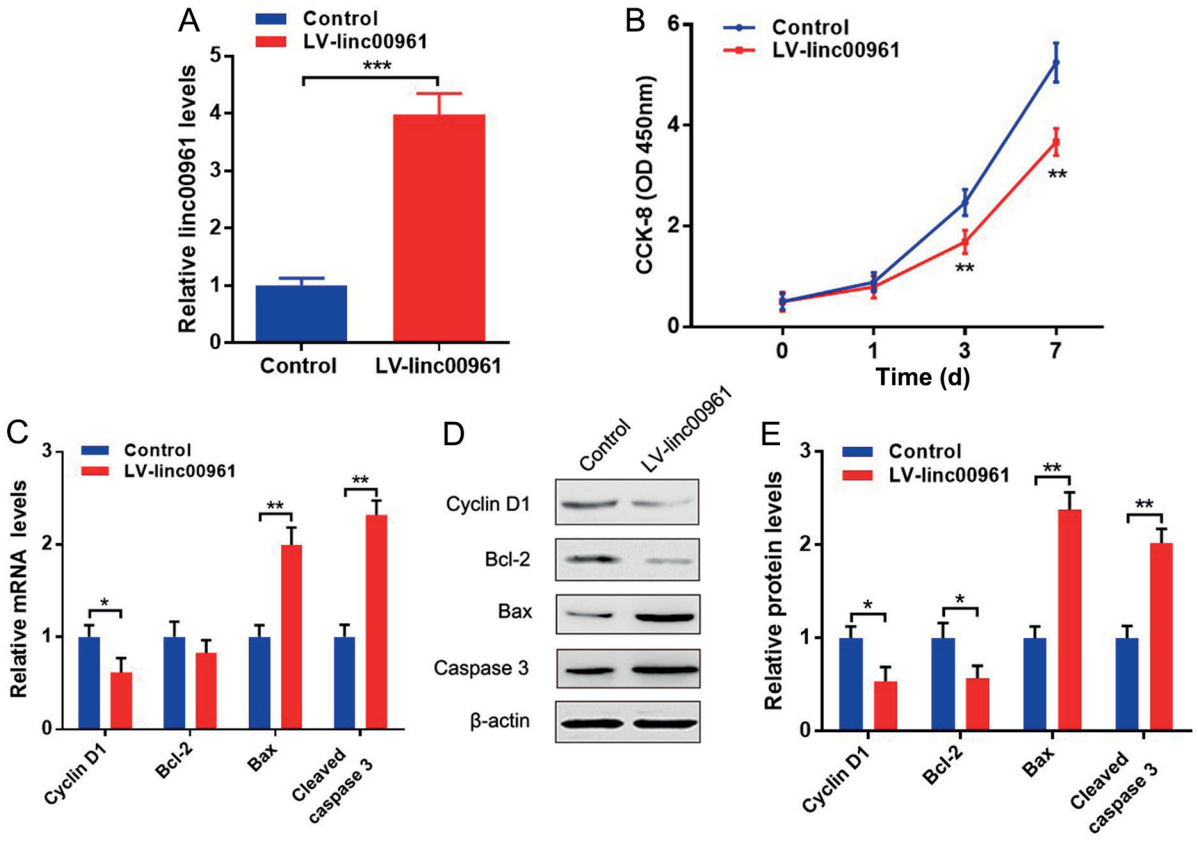

Figure 3. Upregulation of linc00961 inhibited proliferation and promoted apoptosis of VSMCs. **A,** The linc00961 expression was detected after LV-linc00961 infection by RT-PCR. **B,** The proliferation rate of VSMCs was analyzed by CCK-8 assay. **C,-E,** The mRNA and protein levels of Cyclin D1, apoptotic, and anti-apoptotic genes were detected by RT-PCR and WB. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05; **p<0.01, ***p<0.001.

and cleaved caspase-3. The data showed that the levels of Cyclin D1 and Bcl-2 were significantly repressed, while the levels of Bax and cleaved caspase-3 were significantly increased in the LV-linc00961 group (Figure 3C-3E) (p<0.05), compared to the control. These results indicated that the upregulation of linc00961 inhibited proliferation and promoted the apoptosis of VSMCs.

Downregulation of Linc00961 Promoted Proliferation and Inhibited Apoptosis of VSMCs

To further prove the functions of linc00961 in atherosclerosis, the LV-sh linc00961 was constructed, which resulted in linc00961 downregulation. After LV-sh linc00961 infection to VSMCs, the linc00961 level was significantly inhibited (Figure 4A) (p<0.01). The CCK-8 assay showed that the downregulation of linc00961 significantly promoted VSMCs proliferation at the time of 3 d and 7 d, compared with the control group (Figure 4B) (p<0.05). Furthermore, the levels of Cyclin D1 and Bcl-2 were significantly increased, while the levels of Bax and cleaved caspase-3 were significantly inhibited in the LV-sh linc00961 group (Figure 4C-4E) (p<0.05), compared to the control. These results indicated that the downregulation of linc00961 promoted proliferation and inhibited apoptosis of VSMCs.

Linc00961 Could Directly Bind With MiR-367 in VSMCs

To explore the underlying mechanisms of linc00961 that regulated cell proliferation and apoptosis in VSMCs, the bioinformatics analysis was performed, and miR-367 was identified as a potential targeting miRNA by using starBase v2.0 database, which contained the putative binding sites with linc00961. Then, the levels of miR-367 in CHD patients and ApoE-/-mice were detected by RT-PCR. The data obtained showed that miR-367 expressions were significantly in-

creased in both CHD patients and ApoE-/-mice (Figure 5A-5B) (p<0.001). Moreover, the levels of miR-367 were negatively correlated with the levels of linc00961 in CHD patients and ApoE-/-mice (Figure 5C-5D) (p<0.05). Furthermore, we also detected the miR-367 expressions in VSMCs infected with LV-linc00961 and LV-sh linc00961. The findings revealed that miR-367 was significantly downregulated following the upregulation of linc00961, while it was upregulated following the downregulation of linc00961 (Figure 5E-5F) (p<0.01). These results indicated that linc00961 negatively interacted with miR-367, and miR-367 was predicted to be one target of linc00961. To confirm whether linc00961 could function as a ceRNA to competitively bind with miR-367, WT-linc00961 and MUT-linc00961 vectors were constructed to further explore the association between linc00961 and miR-367 in VSMCs, and the Luciferase gene reporter assay was performed (Figure 5G). The relative Luciferase activity in VSMCs co-transfected with WT-linc00961 and miR-367 mimics was significantly repressed compared with the cells transfected with miR-367 NC. However, the relative Luciferase activity in VSMCs co-transfected with MUT-linc00961 and miR-367 mimic was reversed (Figure 5H) (p<0.01). These results indicated that linc00961 could function as a ceRNA to competitively bind with miR-367 in VSMCs.

Linc00961 Repressed Proliferation and Promoted Apoptosis of VSMCs Via MiR-367

To investigate whether linc00961 inhibited proliferation and promoted apoptosis of VSMCs binding with miR-367, the miR-367 inhibitor was added into VSMCs with LV-sh linc00961, and the CCK-8 and apoptotic genes were examined. The results showed that the increased

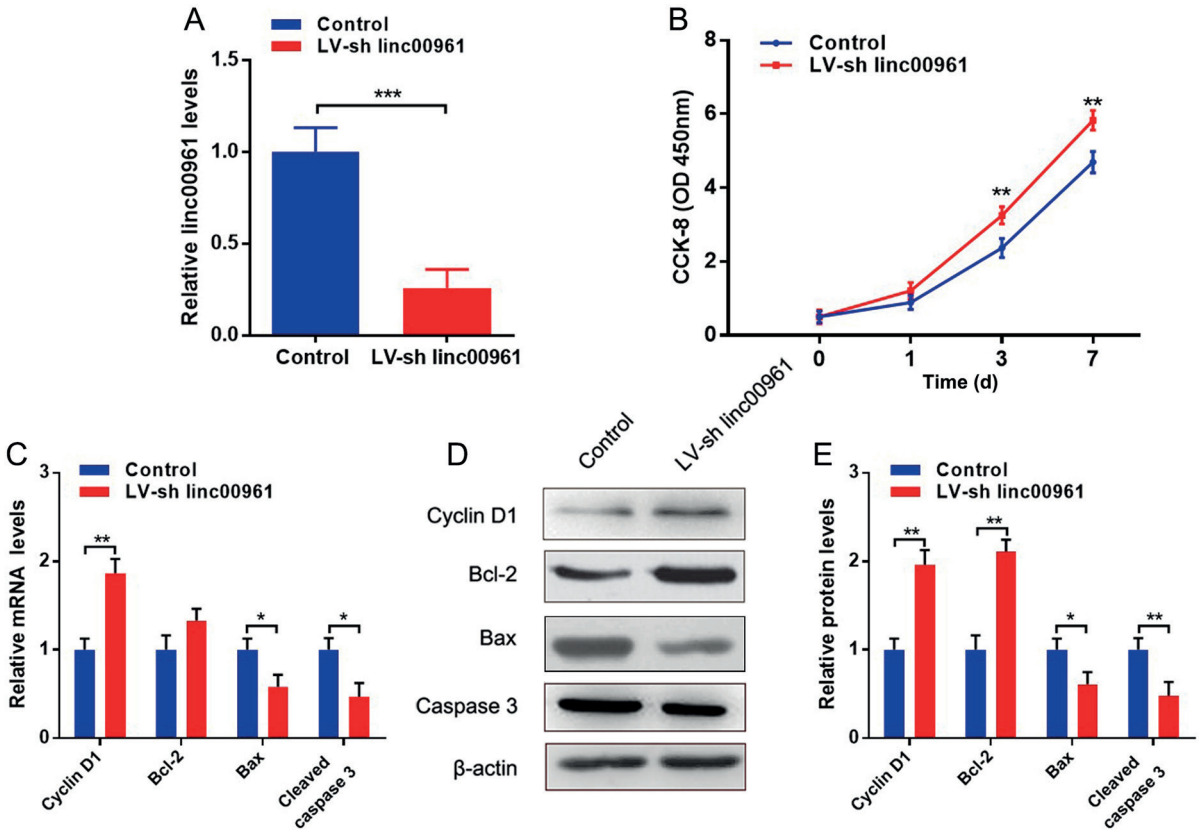

Figure 4. Downregulation of linc00961 promoted proliferation and inhibited apoptosis of VSMCs**. A,** The linc00961 expression was detected after LV-sh linc00961 infection by RT-PCR. **B,** The proliferation rate of VSMCs was analyzed by CCK8 assay. **C,-E,** The mRNA and protein levels of Cyclin D1, apoptotic, and anti-apoptotic genes were detected by RT-PCR and WB. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05; **p<0.01, ***p<0.001.

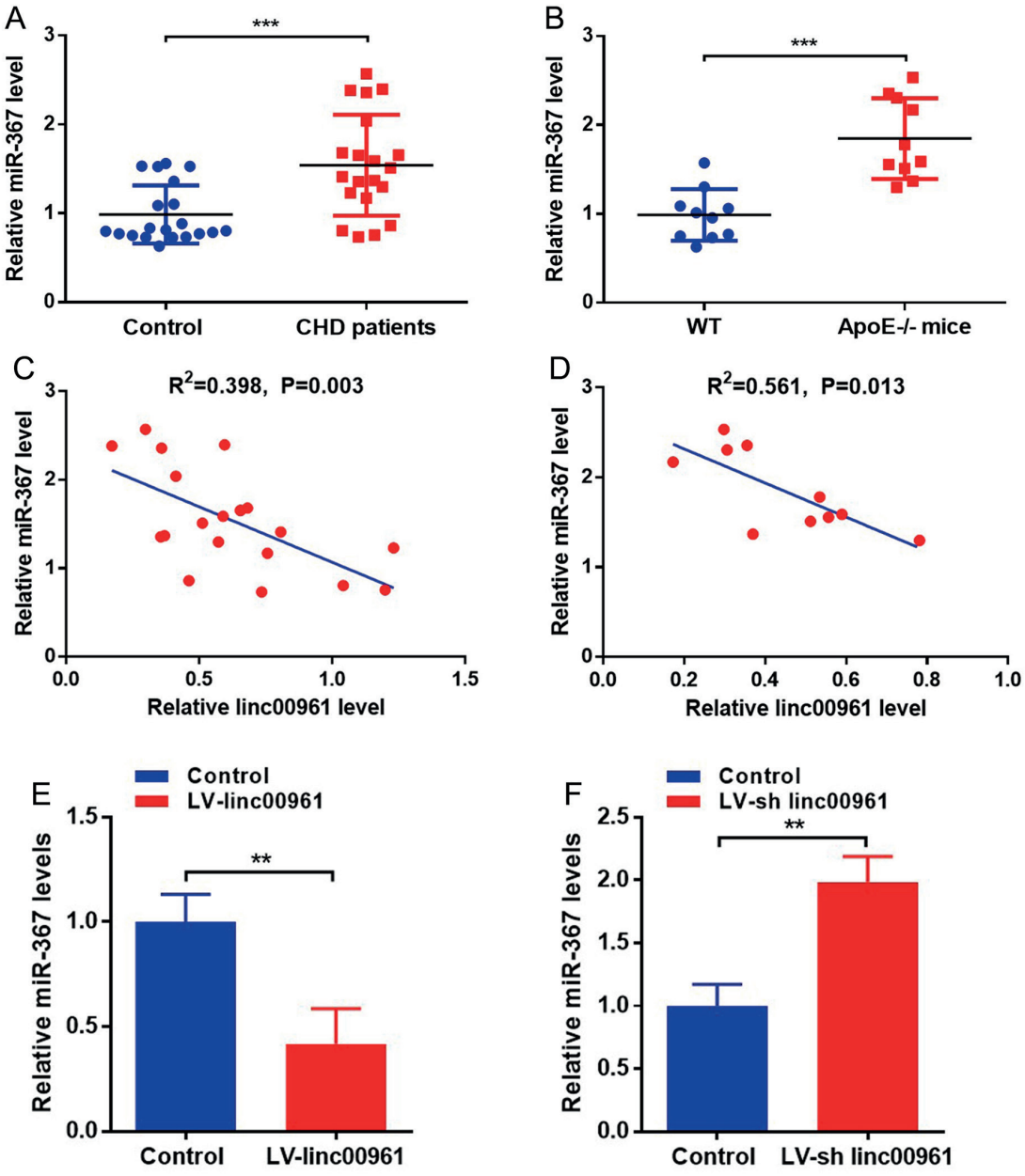

Figure 5. Linc00961 could directly bind with miR-367 in VSMCs. **A-B,** The mRNA levels miR-367 were detected by RT-PCR in CHD patients and ApoE-/-mice. **C-D,** The relationship between linc00961 and miR-367 was analyzed by correlation analysis. **E-F,** The levels of miR-367 were detected by RT-PCR in VSMCs infected with LV-linc00961 and LV-sh linc00961. **G,** Potential binding sites between linc00961 and miR-367 were predicted by starBase v2.0 database. **H,** The Luciferase reporter assay was performed to determine the binding site. Data are shown as mean ± SD based on at least three independent experiments, **p<0.01, ***p<0.001.

cell proliferation rate in VSMCs with LV-sh linc00961 was significantly reversed after adding miR-367 inhibitor at 3 d and 7 d (Figure 6A) (p<0.05). The increased levels of Cyclin D1 and Bcl-2 in VSMCs with LV-sh linc00961 were significantly repressed after adding miR-367 inhibitor, while the repressed levels of Bax and cleaved caspase 3 in VSMCs with LV-sh linc00961 were significantly increased (Figure 6B-6D) (p<0.05). These findings indicated that linc00961 inhibited proliferation and promoted apoptosis of VSMCs *via* binding with miR-367.

Discussion

Atherosclerosis is one of the leading causes of mortality in the world, and it is a multistep cardiovascular disease. It is reported that the proliferation of VSMCs and the formation of neo-intima play an important role in the development of atherosclerosis and CHD[1-3]. LncRNAs have been found to be involved in the regulation and development of cardiovascular diseases, such as atherosclerosis and CHD[21,22]. Linc00961 has been reported to be a tumor suppressor in multiple types of cancers[26-28]. However, the level and biological roles of linc00961 in CHD are less understood. In this study, we found that linc00961 expression was reduced in both CHD patients and ApoE-/-mice, so it might play a role in CHD patients. To evaluate which factor might affect the linc00961 expression, VAMCs were respectively treated with inflammatory factors and hypoxia conditions (4% O_2). We found that the treatments of hypoxia and CRP would decrease the linc00961 expression, which might provide a valuable research orientation for further study. We still did not know its function in CHD and atherosclerosis.

To further investigate the function of linc00961 in CHD, the LV-linc00961, and LV-sh linc00961 were constructed and respectively infected into VSMCs. The results showed that the VSMCs proliferation was repressed in LV-linc00961, the expressions of Cyclin D1 and Bcl-2 were decreased,

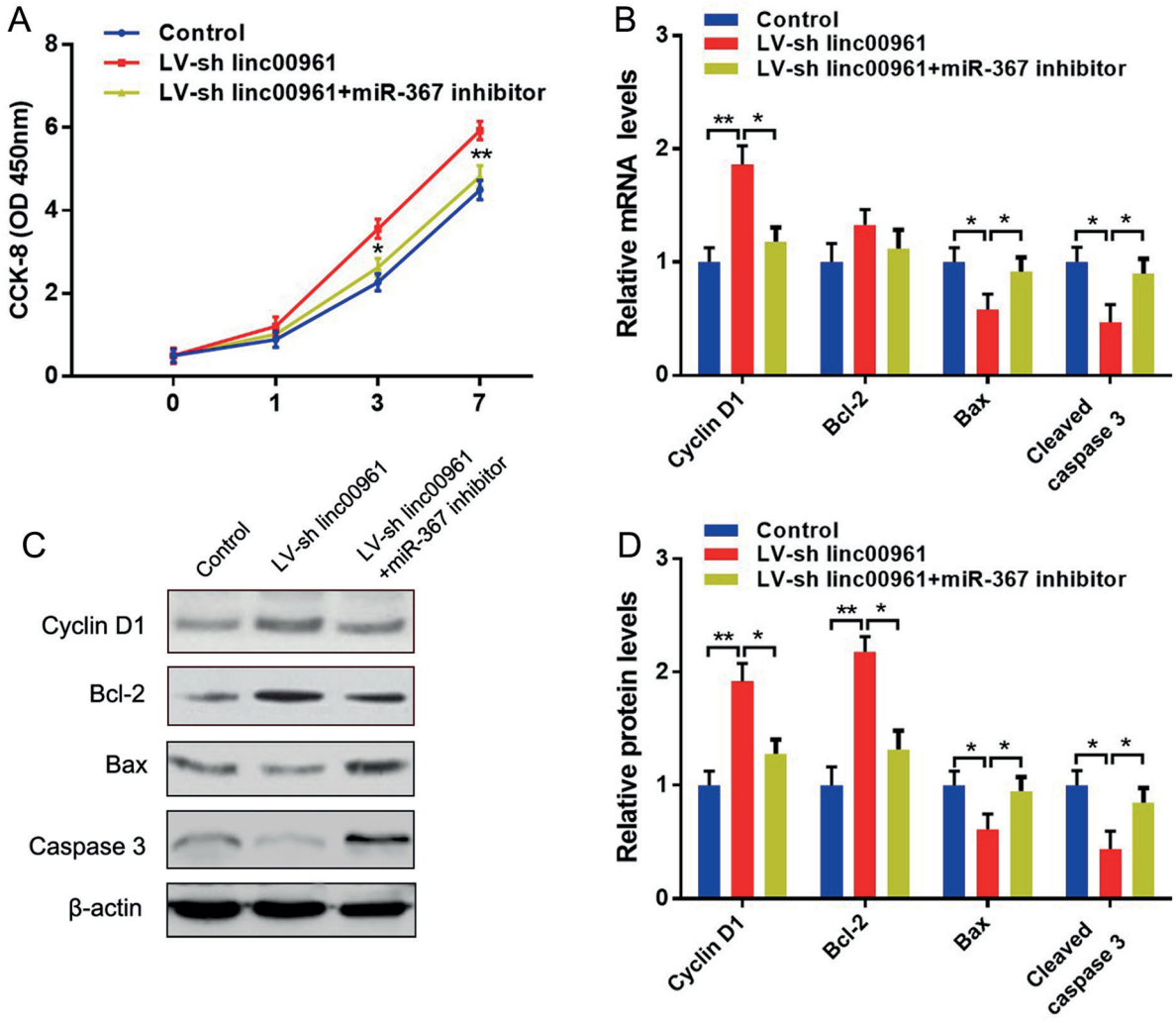

Figure 6. Linc00961 repressed proliferation and promoted apoptosis of VSMCs *via* miR-367. **A,** The proliferation rates of VSMCs were analyzed by CCK-8 assay in VSMCs with LV-sh linc00961 and LV-sh linc00961 with miR-367 inhibitor. **B-D,** The mRNA and protein levels of Cyclin D1, apoptotic, and anti-apoptotic genes were detected by RT-PCR and WB. Data are shown as mean ± SD based on at least three independent experiments, *p<0.05; **p<0.01.

Bax and cleaved caspase-3 were increased. However, these results were reversed in LV-sh linc00961, demonstrating that linc00961 inhibited proliferation and promoted apoptosis of VSMCs. Pan et al[27] reported that linc00961 suppressed cell proliferation and induced cell apoptosis in oral squamous cell carcinoma. Mu et al[26] reported that linc00961 could inhibit the proliferation and invasion of skin melanoma.

To explore the underlying mechanisms of linc00961 that regulated cell proliferation and apoptosis in VSMCs, bioinformatics analysis was performed, and miR-367 was identified as a potential targeting miRNA by using starBase v2.0 database. MiR-367 was demonstrated to be involved in the proliferation and progression of multiple types of cancers[29,30]. Then, we detected the levels of miR-367. The results showed that miR-367 expressions were increased in both CHD patients and ApoE-/-mice, which was negatively correlated with linc00961 expression. To confirm whether linc00961 could function as a ceRNA to competitively bind with miR-367, WT-linc00961 and MUT-linc00961 vectors were constructed, and the Luciferase gene reporter assay was performed. The results showed that linc00961 could directly bind with miR-367, which indicated that linc00961 could function as a ceRNA to competitively bind with miR-367 in VSMCs. To further investigate whether linc00961 inhibited proliferation and promoted apoptosis of VSMCs *via* miR-367, the miR-367 inhibitor was added into VSMCs with LV-sh linc00961. The results showed that the increased cell proliferation rate in VSMCs with LV-sh linc00961 was reversed after adding a miR-367 inhibitor.

Therefore, our study showed that the reduced linc00961 in CHD patients could promote cell proliferation and enhance the development of CHD, suggesting that linc00961 might be used as a new target for CHD and provide a new intervention strategy for patients with CHD, but it should be verified in human CHD patients.

Conclusions

In summary, our study results revealed that linc00961 was significantly decreased in patients with CHD and ApoE-/-mice. Furthermore, our findings firstly uncovered that linc00961 was reduced by hypoxia and CRP in VSMCs. The downregulation of linc00961 contributed to promote proliferation and inhibit apoptosis of VSMCs by sponging miR-367 in CHD patients. Thus, linc00961 might be a promising marker and provide a potential target for treating atherosclerosis.

Conflicts of interest

The authors declare no conflicts of interest.

References

1) Lu Y, Ballew SH, Tanaka H, Szklo M, Heiss G, Coresh J, Matsushita K. 2017 ACC/AHA blood pressure classification and incident peripheral artery disease: the Atherosclerosis Risk in Communities (ARIC) Study. Eur J Prev Cardiol 2019: 2047487319865378. doi: 10.1177/2047487319865378. [Epub ahead of print].
2) Juraschek SP, Daya N, Appel LJ, Miller ER 3rd, Matsushita K, Michos ED, Windham BG, Ballantyne CM, Selvin E. Subclinical cardiovascular disease and fall risk in older adults: results from the atherosclerosis risk in communities study. J Am Geriatr Soc 2019; 67: 1795-1802.
3) Wong MC, Zhang DX, Wang HH. Rapid emergence of atherosclerosis in Asia: a systematic review of coronary atherosclerotic heart disease epidemiology and implications for prevention and control strategies. Curr Opin Lipidol 2015; 26: 257-269.
4) Herrington W, Lacey B, Sherliker P, Armitage J, Lewington S. Epidemiology of atherosclerosis and the potential to reduce the global burden of atherothrombotic disease. Circ Res 2016; 118: 535-546.
5) Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Després JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, Turner MB; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation 2015; 131: e29-e322.
6) Kyaw T, Peter K, Li Y, Tipping P, Toh BH, Bobik A. Cytotoxic lymphocytes and atherosclerosis: significance, mechanisms and therapeutic challenges. Br J Pharmacol 2017; 174: 3956-3972.
7) Nguyen MT, Fernando S, Schwarz N, Tan JT, Bursill CA, Psaltis PJ. Inflammation as a therapeutic target in atherosclerosis. J Clin Med 2019; 8. pii: E1109.
8) Marchio P, Guerra-Ojeda S, Vila JM, Aldasoro M, Victor VM, Mauricio MD. Targeting early atherosclerosis: a focus on oxidative stress and inflammation. Oxid Med Cell Longev 2019; 2019: 8563845.
9) Christen T, Trompet S, Rensen PCN, Willems van Dijk K, Lamb HJ, Jukema JW, Rosendaal FR, le Cessie S, de Mutsert R. The role of inflammation in the association between overall and visceral adiposity and

subclinical atherosclerosis. Nutr Metab Cardiovasc Dis 2019; 29: 728-735.

10) Barranco C. Atherosclerosis linked to faulty DNA repair in VSMCs. Nat Rev Cardiol 2018; 15: 380.

11) Wang C, Zhang Y, Yang Q, Yang Y, Gu Y, Wang M, Wu K. A novel cultured tissue model of rat aorta: VSMC proliferation mechanism in relationship to atherosclerosis. Exp Mol Pathol 2007; 83: 453-458.

12) Lv G, Zhu H, Li C, Wang J, Zhao D, Li S, Ma L, Sun G, Li F, Zhao Y, Gao Y. Inhibition of IL-8-mediated endothelial adhesion, VSMCs proliferation and migration by siRNA-TMEM98 suggests TMEM98's emerging role in atherosclerosis. Oncotarget 2017; 8: 88043-88058.

13) Zhang MJ, Zhou Y, Chen L, Wang X, Long CY, Pi Y, Gao CY, Li JC, Zhang LL. SIRT1 improves VSMC functions in atherosclerosis. Prog Biophys Mol Biol 2016; 121: 11-15.

14) Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? Oncogene 2012; 31: 4577-4587.

15) Hauptman N, Glavac D. MicroRNAs and long non-coding RNAs: prospects in diagnostics and therapy of cancer. Radiol Oncol 2013; 47: 311-318.

16) Morris KV, Mattick JS. The rise of regulatory RNA. Nat Rev Genet 2014; 15: 423-437.

17) Li Y, Wang Z, Nair A, Song W, Yang P, Zhang X, Sun Z. Comprehensive profiling of lincRNAs in lung adenocarcinoma of never smokers reveals their roles in cancer development and prognosis. Genes (Basel) 2017; 8. pii: E321.

18) Zhang S, Han J, Zhong D, Liu R, Zheng J. Genome-wide identification and predictive modeling of lincRNAs polyadenylation in cancer genome. Comput Biol Chem 2014; 52: 1-8.

19) Ding X, Zhu L, Ji T, Zhang X, Wang F, Gan S, Zhao M, Yang H. Long intergenic non-coding RNAs (LincRNAs) identified by RNA-seq in breast cancer. PLoS One 2014; 9: e103270.

20) Zarate R, Boni V, Bandres E, Garcia-Foncillas J. MiRNAs and lincRNAs: could they be considered as biomarkers in colorectal cancer? Int J Mol Sci 2012; 13: 840-865.

21) Wang Y, Song X, Li Z, Liu B. Long non-coding RNAs in coronary atherosclerosis. Life Sci 2018; 211: 189-197.

22) Fasolo F, Gregoli KD, Maegdefessel L, Johnson JL. Non-coding RNAs in cardiovascular cell biology and atherosclerosis. Cardiovasc Res 2019; 115: 1732-1756.

23) Li FP, Lin DQ, Gao LY. LncRNA TUG1 promotes proliferation of vascular smooth muscle cell and atherosclerosis through regulating miRNA-21/PTEN axis. Eur Rev Med Pharmacol Sci 2018; 22: 7439-7447.

24) Ye ZM, Yang S, Xia YP, Hu RT, Chen S, Li BW, Chen SL, Luo XY, Mao L, Li Y, Jin H, Qin C, Hu B. LncRNA MIAT sponges miR-149-5p to inhibit efferocytosis in advanced atherosclerosis through CD47 upregulation. Cell Death Dis 2019; 10: 138.

25) Matsumoto A, Pasut A, Matsumoto M, Yamashita R, Fung J, Monteleone E, Saghatelian A, Nakayama KI, Clohessy JG, Pandolfi PP. mTORC1 and muscle regeneration are regulated by the LINC00961-encoded SPAR polypeptide. Nature 2017; 541: 228-232.

26) Mu X, Mou KH, Ge R, Han D, Zhou Y, Wang LJ. Linc00961 inhibits the proliferation and invasion of skin melanoma by targeting the miR367/PTEN axis. Int J Oncol 2019; 55: 708-720.

27) Pan LN, Sun YR. LINC00961 suppresses cell proliferation and induces cell apoptosis in oral squamous cell carcinoma. Eur Rev Med Pharmacol Sci 2019; 23: 3358-3365.

28) Chen D, Zhu M, Su H, Chen J, Xu X, Cao C. LINC00961 restrains cancer progression via modulating epithelial-mesenchymal transition in renal cell carcinoma. J Cell Physiol 2019; 234: 7257-7265.

29) Xiao G, Gao X, Sun X, Yang C, Zhang B, Sun R, Huang G, Li X, Liu J, Du N, Liu D, Liang R, Ren H, Qin S. MiR-367 promotes tumor growth by inhibiting FBXW7 in NSCLC. Oncol Rep 2017; 38: 1190-1198.

30) Ling JW, Lu PR, Zhang YB, Jiang S, Zhang ZC. MiR-367 promotes uveal melanoma cell proliferation and migration by regulating PTEN. Genet Mol Res 2017; 16. doi: 10.4238/gmr16039067.

31) Long J, Luo J, Yin X. miR-367 enhances the proliferation and invasion of cutaneous malignant melanoma by regulating phosphatase and tensin homolog expression. Mol Med Rep 2018; 17: 6526-6532.

32) Ding D, Zhang Y, Wen L, Fu J, Bai X, Fan Y, Lin Y, Dai H, Li Q, Zhang Y, An R. MiR-367 regulates cell proliferation and metastasis by targeting metastasis-associated protein 3 (MTA3) in clear-cell renal cell carcinoma. Oncotarget 2017; 8: 63084-63095.