The Compatibility of Alisma and Atractylodes Affects the Biological Behaviors of VSMCs Via Inhibiting miR-128-5p / p21 Gene

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Research

Keywords: Alisma and Atractylodes, VSMCs, MiR-128-5p, Atherosclerosis

DOI: https://doi.org/10.21203/rs.3.rs-68415/v1

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Abstract

Background: The compatibility of *Alisma and Atractylodes* (AA) has been estimated to exhibit anti-atherosclerotic effects, while the mechanism still remains unclear. This study was aimed to identify the role of AA on oxidized low density lipoprotein (ox-LDL)-induced vascular smooth muscle cells (VSMCs) behaviors, and to explore the effect of microRNAs (miRNAs).

Methods: Scratch wound healing assay detected the migration of VSMCs, immunocytochemistry assay and western blot of SM22α were used to evaluated the phenotypic transformation. Bromodeoxyuridine (BrdU) immunocytochemistry and flow cytometry were applied to detect the proliferation of VSMCs. The miRNA microarray profiling was performed using lianchuan biological small RNA sequencing analysis. VSMCs were transfected with the miR-128-5p mimic and inhibitor, the migration, phenotypic modulation and proliferation of VSMCs were investigated. The 3’UTR binding sequences site of miR-128-5p on p21 gene were predicted and assessed by luciferase assays.

Results: AA and extracellular regulated protein kinases 1/2 (ERK1/2) blocker U0126 markedly inhibited the ability of migration, elevated smooth muscle 22alpha (SM22α) expression, repressed VSMCs proliferation, elevated the expression of miR-466f-3p and miR-425-3p, while suppressed miR-27a-5p and miR-128-5p expression in the ox-LDL-induced VSMCs. MiR-128-5p targets tissue inhibitor of metalloproteinase (TIMPs), silent information regulator 2 (SIRT2), peroxisome proliferator-activated receptors (PPARs) and p21 genes, which is linked to the behaviors of VSMCs. MiR-128-5p mimic promoted migration and proliferation of VSMCs, and suppressed the p21, p27 and SM22α expression. The inhibitor increased p21, p27 and SM22α expression, and repressed the migration, phenotypic transformation and proliferation of VSMCs. MiR-128-5p directly targeted the 3’UTR binding sequences of p21 gene, negatively regulated p21 expression and exerted a role in the proliferation of VSMCs.

Conclusion: Our research showed that the migration, phenotypic transformation and proliferation of ox-LDL-induced VSMCs were repressed by AA through inhibiting miR-128-5p via targeting p21 gene, which may provide an effective option for the treatment of Atherosclerosis.

Background

Atherosclerosis (AS), the main pathological process of arterial lesions, is the cause of the majority of cardiovascular and cerebrovascular diseases. It has been shown that inflammation caused by oxidized low density lipoprotein (ox-LDL) contributes to the occurrence and development of AS [1], which can promote vascular smooth muscle cells (VSMCs) migration, proliferation and transformation from contractile to synthetic phenotype [2–6]. A large number of cytokines, extracellular matrix (ECM) and matrix metalloproteinases (MMPs) are synthesized and secreted by synthetic VSMCs during the progression of AS [7,8]. The migration, phenotypic transformation and proliferation of VSMCs lead to vascular wall remodeling, which is mediated by activation of the extracellular regulated protein kinases 1/2 (ERK1/2) signaling [9].
MicroRNAs (miRNAs) are small non-coding sequences involved in the negative regulation of gene expression. A series of cellular pathophysiological mechanisms involved in AS, (eg, differentiation, proliferation and signaling pathways), are under control of miRNAs. MiRNAs are recognized as important regulators of lipid metabolism, inflammatory mediators and VSMCs behaviors in the progression of AS [10]. Several studies have shown that miRNAs display a multiple role on the phenotypic transformation, migration and proliferation of VSMCs via inhibiting ERK1/2 activation [11,12], partly through regulating the tissue inhibitor of metalloproteinases (TIMPs)-MMPs and p21-cyclins interaction [13–18].

Alisma and Atractylodes (AA) was one of the classic traditional Chinese medicinal formula first appeared in “Synopsis of Prescriptions of the Golden Chamber”. It has been displayed that AA exhibits multiple pharmacological actions [19–22]. It is reported that Alisma orientale has a protective effect on acute lung injury through anti-inflammatory effect via repressing NF-kappa B pathway [20]. The extract of Alisma orientale exerted a protective role on nonalcoholic fatty liver disease and palmitate-induced cellular injury [21, 22], suggesting that it could be a potential treatment for abnormal lipid metabolism syndrome. Several studies showed that AA repressed lipid deposition in macrophages-derived foam cells [23]. Alisol A 24-Acetate, an active extract derived from Alisma orientale, inhibited the migration and repressed the transformation from contractile to synthetic phenotype in ox-LDL-induced VSMCs via inhibiting ERK1/2 signaling [9]. However, little is known about the mechanisms of AA on VSMCs migration, phenotypic transformation and proliferation. This study was aimed to investigate the impact of AA on ox-LDL-induced VSMCs and explore the underlying mechanisms of miRNAs.

Material And Methods

Ethical statement and Animals

All Sprague-Dawley (SD) rats, provided by Fujian University of Traditional Chinese Medicine (Fujian, China), were performed following the Guidelines Suggestions for the Care and Use of Laboratory Animals 2006 administered by Ministry of Science and Technology, China. This study was approved by Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine (permission number: 2015-016). All rats were housed in facilities and provided food and water ad libitum.

Preparation of AA containing serum

AA-containing serum was prepared according to previous study [23]. In brief, the rats were assigned to 2 groups and then were given AA and 0.9 %NaCl respectively by oral gavage 4 ml twice daily for 7 d. Then, blood samples were collected from the rat abdominal aorta following induction of anesthesia with diazepam/ketamine (1:1) (1 ml/1000 g), and serum was separated by centrifugation at 3000 r·min⁻¹ at 4 °C for 10 min. After filtering the bacteria with a microporous membrane, the serum was inactivated in 56 °C water bath for 30 min, then stored at -20 °C for the study.

VSMCs isolation and culture
VSMCs were prepared from rat as described previously. VSMCs were removed from the rats thoracic aorta and cultivated with Dulbecco's modified Eagle's medium (DMEM)/F12 (GIBCO, Life Technologies, USA) and 20% fetal bovine serum (GIBCO, Life Technologies, USA) containing penicillin and streptomycin solution (1:1) (GIBCO, Life Technologies, USA) at 37 °C with 5% CO₂. VSMCs grown to 85%–90% confluence were forced quiescent by FBS-free serum starvation for 24 h. Then VSMCs were assigned into four groups. Control group: VSMCs were cultured in DMEM/F12 with 20% normal rat serum; ox-LDL group: VSMCs were cultured with 20% normal rat serum and 50 mg·L⁻¹ ox-LDL (Peking Union-Biological Co, Ltd.; Beijing, China); AA group: VSMCs were cultured in DMEM/F12 addition with 20% AA and 50 mg·L⁻¹ ox-LDL; U0126 group: VSMCs were treated with 10 μmol·L⁻¹ U0126 (Sigma-Aldrich, Inc.; St. Louis, MO, USA) and 50 mg·L⁻¹ ox-LDL.

**VSMCs migration assay**

VSMCs migration was assayed by Scratch Wound Healing. Well-functioning VSMCs (1.0×10⁵ cells/well) at 60-70% confluence were incubated in a 6-well dish and starved with DMEM/F12 containing 0.5% FBS for 12 h. A linear wound was scratched the center of the cell monolayer with a 200 ul pipette tip and washed with PBS twice. VSMCs in the assigned groups were treated as described above in DMEM/F12 with 20% FBS. Cells were cultured 24 h at 37 °C with 5% CO₂, and then the images of scratches were observed using a Leica DMIL LED inverted microscope (Wetzlar, Germany) and LAS Interactive Measurement imaging analysis software (Leica Microsystems, Mannheim, Germany).

**Detection of phenotypic transformation in VSMCs**

Immunofluorescence assay was used to detect the expression of the VSMCs phenotypic marker smooth muscle 22α (SM22α). Logarithmic-phase VSMCs (1×10⁵ cells/well) were incubated onto a cover glass in a 6-well plates. VSMCs in the assigned groups were treated for 24 h as described above. VSMCs were fixed in 4% paraformaldehyde for 15 min, rinsed with PBS, passed through 0.3% TritonX-100 for 15 min, blocked with goat serum for 30 min, co-cultured with anti-Rabbit SM22α monoclonal antibody (1:200; proteintech, USA), rinsed with PBS, incubated with secondary antibody (1:200; proteintech, USA), and DAPI dye solution (100 ng·ml⁻¹, BOSTER Biological Technology, China), drain the dye solution and observed under fluorescence microscope. Five fields of vision were randomly photographed from each well plates, and comprehensively analyzed using the imaging processing software (Image-Pro Plus v6.0, Media Cybernetics; Bethesda, Md., USA).

**MMP-2 and MMP-9 ELISA**

The secretion of MMP-2 and MMP-9 in the VSMCs were detected by ELISA. The cells were treated for 24 h as described above and the culture supernatants were harvested. According to the manufacturer's instructions, the expression of MMP-9 and MMP-2 were measured in the cell culture supernatant using a sandwich ELISA kit (BOSTER Biological Technology, China).
VSMCs proliferation assay

BrdU immunocytochemistry and flow cytometry were used to measure the proliferation of VSMCs. VSMCs (1×10^5 cells/well) were seeded onto 6-well plates and incubated in DMEM/F12 supplemented with 20% FBS for 24 h. Quiescent VSMCs at 60-70% confluence in DMEM/F12 with 0.5% FBS were grouped as described above and cultivated with BrdU (30 μmol/L) for 24 h. Follow the instructions of the FITC-brdU cell proliferation assay kit (BOSTER Biological Technology, China), detected by flow cytometry, 488 nm excitation wavelength, 520 nm Emission wavelength. BrdU expression was performed by immunocytochemical staining following the manufacturer's instructions. Five fields of vision were randomly photographed from each well plates, and comprehensively analyzed using the imaging processing software (Image-Pro Plus v 6.0, Media Cybernetics; Bethesda, Md., USA). The positive cells were detected by BrdU manifesting pale-yellow or deep-tan, while negative manifesting nonspecific background staining.

MiRNA microarray assay and miRNA target genes interaction

Total RNA was extracted from VSMCs. The miRNA microarray profiling was performed using Lianchuan Biological Small RNA Sequencing Analysis (Lianchuan Bio, China) according to manufacturer's recommended protocol. Small RNA sequencing library preparation was performed using the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, USA). After the library preparation work was completed, the constructed library was sequenced using Illumina Hiseq 2000/2500, and the sequencing read length was single-ended 1×50 bp. The potential target genes of miRNAs were searched with Targetscan7.2 database and miRDB. The target genes interaction of miRNAs were made according to the database.

Transfection of miR-128-5p mimic and inhibitors in VSMCs

VSMCs were kept completely synchronous with serum-starved for 24 h before transfection. VSMCs (2-3×10^5 cells/well) at 60%-70% confluence were treated for 24 h as described above and transfection with 50 nM of miR-128-5p mimic, miR-128-5p inhibitor, and mimic negative control (NC), inhibitor NC using siRNA-Mate plus (GenePharma, Shanghai, China) according to the manufacturer's protocol. The miR-128-5p mimic, inhibitor, and mimic NC, inhibitor NC were designed and synthesized by GenePharma (Shanghai, China). Sequences were as follows: 5′-UCAGUGCUACGGCCCGGUU-3′ (miR-128-5p mimic); 5′-UUCUCGAACGUGACGUTT-3′ (miR-128-5p mimic NC); 5′-UCUCCAGUCUGACGCCC-3′ (miR-128-5p inhibitors); 5′-CAGUACUUUUGUGUAGUACAA-3′ (miR-128-5p inhibitors NC). Total RNA was isolated from VSMCs after transfection using TRizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s manual. The miRNAs expression were detected qRT-PCR with One Step SYBR® PrimeScript™ RT-PCR Kit II (Takara, Tokyo, Japan) according to the manufacturer's protocol.

Western blot determines protein expression of p21, p27 and SM22α

VSMCs after treatment for 24 h as described were collected and lysed with RIPA buffer (Tris-HCl: 50 mM (pH8.0), NP-40:1.0%, Na-deoxycholate:1.0% NaCl:150 mM, SDS:0.1%, PMSF:0.05 mM), and the protein
concentration was assessed using the BCA protein Assay Kit (Abcam, Cambridge, UK). Equal amounts of protein lysates mixed evenly with 6 × loading buffer (5:1, V/V), were transferred to PVDF membrane. After blocked in 5% BSA for 2 h, the membranes were incubated with p21, p27 (1:1000, Cell Signaling Technology, Inc.; Danvers, MA, USA), SM22α, (1:1000, Abcam, Cambridge, UK), mouse anti-rat/rab β-actin (1:1000, Cell Signaling Technology, Inc.; Danvers, MA, USA) at 4 °C overnight. Then the membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 to 2 h and detected chemiluminescent autorography using an ECL kit (Beyotime Biotechnology; Beijing, China). Gray Value of the protein bands were analyzed using the image processing software Image-Lab version 5.0 (Bio-Rad; Hercules, CA, USA).

Luciferase assay

To verify miR-128-5p targeting p21 gene, wild-type (wt) and mutant-type (mut) 3′UTR sequences binding sites of p21 gene were cloned into PGL3-CMV-LUC-MCS vectors using XhoI, MluI restriction sites. HEK-293 cells were co-transfected with pRL-TK vectors, pGL3 vector control, miR-128-5p mimic or mimic-negative controls by using lipofectamine® 2000 (Invitrogen, CA, USA). After incubated for 48 h, firefly luciferase activity was detected using dual-luciferase assays (Genomeditech, Shanghai, China). It was normalized to a Renilla luciferase expression control.

Statistical analysis

The results were presented as Mean±SD. It was performed using the statistical software SPSS version 21.0 and assessed by one-way analysis of variance (ANOVA) or two-tailed Student’s t test to compare two treatments. p value < 0.05 is considered statistically significant.

Results

AA and U0126 suppress the migration of ox-LDL-induced VSMCs

Wound healing assay was used to evaluate the role of AA on VSMCs migration. The control group had a small number of VSMCs migration, and ox-LDL (50 mg·L⁻¹) treatment significantly promoted cells migration (*P < 0.05, Fig. 1). However, both AA and U0126 (10 µmol·L⁻¹) treatment remarkably suppressed the ability of migration in ox-LDL-induced VSMCs (# P < 0.05, Fig. 1).

AA and U0126 elevate the SM22α Expression in ox-LDL-induced VSMCs

The SM22α is a marker protein for the contractile phenotype. Immunofluorescence assay revealed that ox-LDL treatment reduced the mean optical density of SM22α expression (**P < 0.01, Fig. 2). Treatment with AA and U0126 caused elevation of SM22α expression in ox-LDL-induced VSMCs (# P < 0.05, ## P < 0.01, Fig. 2), indicating that AA and U0126 treatment could inhibit the conversion of VSMCs from contractile to synthetic phenotype induced by ox-LDL.

AA and U0126 suppress ox-LDL-induced MMP-2 and MMP-9 secretion in VSMCs
MMP-2 and MMP-9 have shown to be involved in cell migration and proliferation. ELISA was used to detected the expression of MMP-9 and MMP-2 in supernatant of cell culture. Ox-LDL treatment significantly induced the expression of MMP-2 and MMP-9 compared with the control group (**\(P < 0.01\), Fig. 3). However, treatment with AA and U0126 significantly repressed the MMP-2 and MMP-9 expression in Ox-LDL-induced VSMCs (#\(P < 0.05\), ##\(P < 0.01\), Fig. 3).

Aa And U0126 Inhibit Ox-ldl-induced Vsmcs Proliferation

Flow cytometry indicated that ox-LDL increased the number of BrdU positive cells (new proliferation of VSMCs) (*\(P < 0.05\), Fig. 4), while AA and U0126 treatment decreased the number of BrdU positive VSMCs at 24 h (#\(P < 0.05\), ##\(P < 0.01\), Fig. 4), showing that AA and U0126 could inhibit the proliferation of cells.

Aa And U0126 Regulate Micrornas Expression In Ox-ldl-induced Vsmcs

To determine whether miRNAs were involved in the effect of AA on ox-LDL-induced VSMCs, miRNA microarrays analysis was applied to detect the miRNAs expression in ox-LDL-induced VSMCs. MiRNA microarrays assay demonstrated that there were various miRNAs expression among groups, ox-LDL treatment up-regulated 7 miRNAs and down-regulated 4 miRNAs in VSMCs comparing to control group (\(P < 0.01\), Fig. 5a and 5b). AA dramatically inhibited the 6 miRNAs overexpression and up-regulated 10 miRNAs in ox-LDL-induced VSMCs (\(P < 0.01\), Fig. 5a and 5b). ERK1/2 blocker U0126 treatment up-regulated 9 miRNAs and down-regulated 25 miRNAs in ox-LDL-induced VSMCs (\(P < 0.01\), Fig. 5a and 5b). Furthermore, we found that ox-LDL down-regulated the expression of miR-466f-3p and miR-425-3p, while AA and U0126 treatment reversed the tendency (Table 1). The over-expression of miR-27a-5p and miR-128-5p in ox-LDL-induced VSMCs were dramatically inhibited by treatment with AA and U0126 (Fig. 5a and 5b, Table 1). The sequences of miRNAs were showed in Table 2. Target genes of miRNAs were analyzed according to Targetscan 7.2 database and miRDB. Both miR-466f-3p and miR-425-3p negatively regulates cyclins, cyclin-dependent kinases (CDKs) and MMPs. Peroxisome proliferator-activated receptor(PPAR)\(\delta\), PPAR\(\delta\), p21, silent information regulator 2 (SIRT2), TIMP4 and TIMP3 are the target genes of miR-128-5p and miR-27a-5p, indicating that AA could alter the miRNAs expression in ox-LDL-induced VSMCs and regulate the expression of MMPs-TIMPs, p21-cyclins as well as ERK1/2 inhibitor, which is related with migration, phenotypic transformation and proliferation of VSMCs (Fig. 5c).
Table 1
Individual member of miRNAs expression

| miRNAs   | Control   | ox-LDL    | AA         | U0126     |
|----------|-----------|-----------|------------|-----------|
| miR-466f-3p | 58.33 ± 3.21 | 25.33 ± 1.53## | 42.00 ± 6** | 34.00 ± 1.33@ |
| miR-425-3p  | 276.33 ± 20.30 | 198.33 ± 24.68## | 244.00 ± 15.00* | 260.00 ± 20.42@ |
| miR-27a-5p  | 907.33 ± 5.57    | 1,091.33 ± 96.60## | 736.00 ± 10.15** | 568.00 ± 80.00@@ |
| miR-128-5p  | 45.33 ± 9.02     | 74.67 ± 6.81## | 48.67 ± 10.60** | 40.00 ± 6.71@@ |

#P< 0.05, ##P< 0.01 versus Control; *P< 0.05, **P< 0.01 versus ox-LDL; @P< 0.05, @@P< 0.01 versus ox-LDL.

Table 2
The sequences of individual member in miRNAs

| miRNAs   | sequences             |
|----------|-----------------------|
| miR-466f-3p | TACACACACACATACACACAGA |
| miR-425-3p  | CATCGGGAATATCGTGTCCGCC   |
| miR-27a-5p  | AGGGCTTAGCTGCTTGTGAGCA     |
| miR-128-5p  | CGGGGCCGTAGCACTGTCTGAGCA     |

The miR-128-5p promotes the migration, phenotypic transformation and proliferation of VSMCs

Due to miR-128-5p negatively regulating p21, PPARs, SIRT2 and TIMPs expression, we assumed that miR-128-5p could influence the migration, phenotypic transformation and proliferation of VSMCs. We transfected the miR-128-5p mimic and inhibitor to test the role of miR-128-5p on biological behaviors of VSMCs. The level of miR-128-5p expression was detected by qRT-PCR. MiR-128-5p mimic promoted the expression of miR-128-5p (*P< 0.05), miR-128-5p inhibitor suppressed levels of miR-128-5p expression (#P< 0.05) (Fig. 6a). Scratch wound healing assay showed that miR-128-5p mimic promoted the migration of VSMCs (*P< 0.05, Fig. 6b), and the inhibitor reduced the migration rate of VSMCs compared with the NC groups (#P< 0.05, Fig. 6b). Immunofluorescence assay and WB were applied to detect the SM22α expression in VSMCs. The mimic suppressed the SM22α expression (*P< 0.05, Fig. 7), the inhibitor elevated SM22α fluorescent expression in VSMCs (#P< 0.05, Fig. 7) compared with NC groups. The same results were confirmed by WB that the mimic inhibited the SM22α expression (*P< 0.05, Fig. 8a), the inhibitor raised the SM22α expression (#P< 0.05, Fig. 8a), suggesting that miR-128-5p overexpression could induce VSMCs transformation from contraction to synthetic phenotype.
The effect of miR-128-5p on the proliferation of VSMCs was detected by WB and immunocytochemistry staining of incorporated BrdU. The results showed that the miR-128-5p mimic suppressed the p21 and p27 expression and the inhibitor raised the p21 and p27 expression compared to NC groups (*\(P < 0.05\), #\(P < 0.05\), respectively, Fig. 8a). It suggests that miR-128-5p can influence cell cycles and affect VSMCs proliferation. Immunocytochemistry staining of incorporated Brdu displayed that the miR-128-5p mimic increased the number of proliferation of VSMCs, and the inhibitor repressed the proliferation of VSMCs compared to NC groups (*\(P < 0.05\), #\(P < 0.05\), respectively, Fig. 8b). It turned out that over-expression of miR-128-5p induced the proliferation of VSMCs, while the inhibitor reversed the results. Furthermore, both AA and U0126 treatment reduced the number of proliferation of VSMCs induced by mimic transfection compared with mimic group (\(\Delta P < 0.05\), Fig. 8b), minting that inhibition of the miRNAs could be a promising therapy for VSMCs proliferation and AA suppressed the VSMCs proliferation through repressing miR-128-5p expression as well as ERK1/2 inhibitor.

**P21 is the direct target of miR-128-5p**

Based on Targetscan ([http://www.targetscan.org/vert_71/](http://www.targetscan.org/vert_71/)) and miRDB ([http://mirdb.org/](http://mirdb.org/)), p21 gene is a potential target of miR-128-5p (Fig. 9a). To explore the role of miR-128-5p on the regulation of p21 expression, HEK-293 cells were co-transfected with a Renilla luciferase reporter vector containing the wild-type (wt) p21 3’UTR and mutant (mut) p21 3’UTR, UTR NC and mimic NC. Our results displayed that firefly luciferase activity in p21 3’UTR (wt) transfected cells was lower than those in control groups (*\(P < 0.05\), Fig. 9b). However, there was less effect of miR-128-5p on the luciferase activity in cells containing mutant p21 3’UTR (mut) (Fig. 9b). Our results demonstrate that miR-128-5p directly binds to the 3’UTR sequence sites of p21 gene.

**Discussion**

As an ancient classical traditional Chinese medicinal formula, the compatibility of *Alisma orientalis* and *Atractylodes macrocephala*, was firstly described in the Eastern Han Dynasty, and exhibited a wide range of bioactivities in diverse cells [9, 19–23]. Until now, its effect on the migration, phenotypic transformation and proliferation of VSMCs induced by ox-LDL remain unclear.

Several studies have been reported that phenotypic transformation of VSMCs is a crucial process and promotes VSMCs proliferation and migration in the process of AS [5]. Our study verified that the abilities of migration, phenotypic transformation and proliferation in ox-LDL-treated VSMCs were higher than that in control group. Previous studies demonstrated that AA could inhibit the phosphorylation of ERK1/2 [9], and AA suppressed the abilities of migration, transformation and proliferation on ox-LDL-induced VSMCs as well as ERK1/2 blocker in the present study, suggesting that AA could affect the biological behaviors of VSMCs, which is associated with the inhibition of ERK1/2 signaling pathway.

MiRNAs are conserved, small and single-stranded noncoding RNAs that regulate negatively gene expression at the post-transcriptional level and therefore repress protein expression. Accumulating
evidences reveal that miRNAs serve as important regulators of a range of behaviors of VSMCs and are involved in molecular signaling pathways of AS [10–12]. In the present study, ox-LDL induced over-expression of miR-27a-5p, miR-128-5p and down-regulated the miR-466f-3p, miR-425-3p expression. AA and U0126 inhibited the expression of miR-27a-5p and miR-128-5p, elevated the miR-466f-3p and miR-425-3p expression in the ox-LDL-induced VSMCs. Previous study showed that the main active ingredient of AA inhibited ERK1/2 phosphorylation [9], suggesting that AA could inhibit ox-LDL-induced VSMCs migration, transformation and proliferation through regulating these miRNAs expression, which could be associated with the ERK1/2 pathway.

Cyclins are recognized as the important mediators in the process of cell cycle [24]. As negative regulator of cyclins, p27 and p21 are found to exert a central role in the cell cycle arrest [25, 26]. The activity of cyclin-dependent kinases (CDKs) is strongly linked to the expression of cyclins, which can initiate DNA synthesis, promote cell cycle, play an important role in cell proliferation [27–29]. P21 prevents DNA replication from inhibiting cell proliferation through the control of CDK2, CDK4 and CDK6. Cyclins and CDKs are the target genes of miR-466f-3p and miR-425-3p, p21 is the target gene of miR-128-5p according to Targetscan7.1 database. Our study showed that miR-128-5p directly targeted the 3'UTRs of p21 gene, suppressed p21 expression and induced the proliferation of VSMCs. Inhibition of miR-128-5p increased the level of p21 and p27 expression, and then inhibited the ability of proliferation. AA and U0126 treatment suppressed the increasing number of VSMCs induced by the miR-128-5p over-expression. Treatment with AA and ERK1/2 inhibitor effectively suppressed the proliferation of VSMCs through inhibiting miR-128-5p in the ox-LDL-induced VSMCs, indicating that AA could inhibit VSMCs proliferation by suppressing miR-128-5p expression, which is closely linked to ERK1/2 signal pathway.

TIMPs plays a central role in suppressing the activation of MMPs. TIMPs-MMPs have been identified as the key molecular in vascular remodeling and are linked to VSMCs proliferation and migration [30–33]. SM22α is a contractile marker of VSMCs, it affects the proliferation of VSMCs and the development of AS[5]. We detected that treatment with AA and U0126 effectively increased the expression of SM22α and suppressed the levels of MMP-2 and MMP-9 in VSMCs exposed to ox-LDL. It is reported that several miRNAs contribute to a SMC-specific transcriptional program in regulating the VSMCs phenotypic transformation and proliferation [34]. In the present study, AA and U0126 obviously suppressed the expression of miR-27a-5p and miR-128-5p, elevated miR-466f-3p and miR-425-3p expression in ox-LDL-induced VSMCs. MMP-11, MMP-13 and MMP-19 are negatively regulated by miR-466f-3p and miR-425-3p. TIMP-3, TIMP-4, PPARγ, PPARδ and SIRT2 are the targeting genes of miR-27a-5p and miR-128-5p according to Targetscan7.1 database. Inhibition of SIRT2 represses the proliferation and synthetic phenotypic transformation of VSMCs [35]. Activation of PPARγ and PPARγ can attenuate VSMCs proliferation and migration [36–38] and exert the protective role on AS [39]. Furthermore, MiR-27a and miR-128 suppress LDL receptor and dysregulate the cholesterol homeostasis which is involved in cholesterol efflux and repress progression of AS [40, 41]. Therefore, we assumed that AA could suppress VSMCs migration, phenotypic transformation and proliferation through altering these miRNAs expression, and exert the multiple roles in the process of AS.
It has been reported that overexpression of miR-128 can significantly decrease VSMCs migration, phenotypic transformation and proliferation by targeting Kruppel-like factor 4 (KLF4) [42]. KLF4 acts as a key repressor of VSMCs differentiation, modulating the expression of SM22α and PPARs [42, 43]. MiR-128-5p has the similar function of miR-128 and TIMP-3, TIMP-4, SIRT2, PPARδ, PPARδ and p21 are the negatively controlled by miR-128-5p. We supposed that miR-128-5p could affect the ability of migration, phenotypic transformation and proliferation in VSMCs. The results displayed that miR-128-5p mimic increased the ability of migration, phenotypic transformation and proliferation in VSMCs. The inhibition of miR-128-5p elevated the SM22α expression, suppressed the migration and proliferation of VSMCs, hinting that inhibition of miR-128-5p may be a promising treatment for the progression of AS. AA and U0126 treatment reduced the increasing number of proliferation in VSMCs induced by miR-128-5p mimic transfection compared with mimic group, indicating that AA and U0126 could inhibit the VSMCs proliferation through repressing miR-128-5p expression. P21 protein is involved in cell proliferation and the 3'UTR of p21 gene contains the binding sequences sites of miR-128-5p. Luciferase assays showed that p21 was the direct target gene of miR-128-5p. MiR-128-5p mimic induced over-expression of miR-128-5p, blocked p21 expression and increased VSMCs proliferation. AA and ERK1/2 blocker suppressed the VSMCs proliferation via repressing miR-128-5p expression. It indicates that miR-128-5p can effectively inhibit the proliferation of VSMCs via targeting p21 expression and AA alters the behaviors of VSMCs through arresting miR-128-5p expression via targeting p21 gene.

Conclusion

In conclusion, the results of our study verify that treatment with AA and ERK1/2 blocker inhibit ox-LDL-stimulated VSMCs migration, phenotypic transformation and proliferation through regulating the expression of miR-466f-3p, miR-425-3p, miR-27a-5p and miR-128-5p, especially by suppressing miR-128-5p via targeting p21 gene. It provides new insights that AA regulates the biological behaviors of VSMCs, and exerts a multiple role in the process of AS.

Abbreviations

AA
Alisma and Atractylodes; ox-LDL: oxidized low density lipoprotein; VSMCs: Vascular smooth muscle cells; miRNAs: microRNAs; Brdu: Bromodeoxyuridine; ERK1/2: Extracellular regulated protein kinases 1/2; SM22α: smooth muscle 22alpha; TIMPs: Tissue inhibitor of metalloproteinase; SIRT2: Silent information regulator 2; PPARs: Peroxisome proliferator-activated receptors; AS: Atherosclerosis; ECM: Extracellular matrix; MMPs: Matrix metalloproteinases; CDKs: cyclin-dependent kinases; KLF4: Kruppel-like factor 4.

Declarations

Acknowledgments
The authors would like to thank the Animal Experimental Center and laboratory of Fujian University of Traditional Chinese Medicine for technical support.

Author Contributions

XXH and WW conceived and designed the research. XXH and WW drafted the manuscript. SJL, LL and WW performed the experiments. LXR and LTT analyzed the data. YZ, LG, XXH and WW edited the article. All authors read and approved the final version of the article.

Funding

This study was supported by the grants from the National Natural Science Foundation of China (Grant No. 81473744 and 81774380). No international grant was used.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon request.

Ethics approval and consent to participate

This study was approved by Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine (permission number: 2015-016).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures
Effects of AA and U0126 on the migration of VSMCs. VSMCs were pretreated with ox-LDL (50 mg·L⁻¹), AA and U0126 (10 μmol·L⁻¹) for 24 h. 3 independent experiments were repeated. Results are presented as the Mean±SD (n=3). *P<0.05 versus control group; #P<0.05 versus ox-LDL group.
Figure 2

Effects of AA and U0126 on the expression of SM22α protein in VSMCs treated with ox-LDL. Data are representative of 3 experiments. Following treatment with ox-LDL (50 mg·L⁻¹), AA and U0126 (10 μmol·L⁻¹) for 24h, SM22α expression was detected by immunofluorescence(n=5). **P<0.01 versus control group; #P<0.05, ##P<0.01 versus ox-LDL group.
Figure 3

AA and U0126 inhibit ox-LDL-induced up-regulation of MMP-2 and MMP-9 in VSMCs. 6 independent experiments were repeated. ELISA detected the secreted MMP-9 and MMP-2 expression in the culture medium (n=6). **P <0.01 versus control group; #P<0.05, ##P<0.01 versus ox-LDL group.

Figure 4

AA and U0126 repress ox-LDL-induced VSMCs proliferation. BrdU postive cells and total cells was calculated. These data are representative of 3 experiments. Results are described as the mean±SD (n=3). *P<0.05 versus control group; #P<0.05, ##P<0.01 versus ox-LDL group.
Figure 5

AA and U0126 regulate microRNAs expression in ox-LDL-induced VSMCs. (a) MiR-466f-3p, miR-425-3p, miR-27a-5p and miR-128-5p are significantly associated with AA and U0126 treatment in the ox-LDL-induced VSMCs (P<0.01). (b) MiRNA microarrays assay show that AA and U0126 regulate various different miRNAs expression in ox-LDL-induced VSMCs. (c) According to Targetscan7.1 database and miRDB, PPARs, p21, SIRT2, TIMPs are potential targets of miR-128-5p and miR-27a-5p, cyclins, CDKs and MMPs are the potential targets of miR-466f-3p and miR-425-3p.
Figure 6

Transfection of miR-128-5p mimic and inhibitor in VSMCs. (a) The expression of miR-128-5p was detected by qRT-PCR. These data are representative of 6 experiments. Results are described as the Mean ± SD (n=6). *P<0.05 versus mimic NC, #P<0.05 versus inhibitor NC. (b) The migration of VSMCs were detected after transfection. These data are representative of 6 experiments. Results are described as the Mean ± SD (n=6). *P<0.05 versus mimic NC, #P<0.05 versus inhibitor NC.
Figure 7

Transfection of miR-128-5p affect phenotypic transformation. The SM22α expression was detected by immunofluorescence assay. These data are representative of 3 experiments. Results are described as the Mean ± SD (n=3). *P<0.05 versus mimic NC, #P< 0.05 versus inhibitor NC.

Figure 8
Transfection of miR-128-5p mimic and inhibitor affect SM22, p21 and p27 expression and proliferation of VSMCs. (a) Western blot was performed to determine the expression of sm22, p21 and p27. These data are representative of 3 experiments. Results are described as the Mean ± SD (n=3). *P<0.05 versus mimic NC, #P<0.05 versus inhibitor NC. (b) Immunocytochemistry staining of incorporated BrdU in VSMCs was performed to investigate the proliferation of VSMCs. These data are representative of 3 experiments. Results are described as the Mean ± SD (n=3). *P<0.05 versus mimic NC, #P<0.05 versus inhibitor NC, ΔP< 0.05 versus mimic NC.

Figure 9
P21 gene is the direct target of miR-128-5p. (a) The predicted miR-128-5p binding sequences in p21 gene and the designed mut sequence (p21-mut). (b) MiR-128-5p mimic was co-transfected with the wild-type or mutated p21 3'UTR sequence vectors in HEK 293T cells. The relative firefly luciferase activity is calculated. 3 independent experiments were repeated. *P<0.05 versus respective NC.