Original Article

Differentially expressed mRNAs and their upstream miR-491-5p in patients with coronary atherosclerosis as well as the function of miR-491-5p in vascular smooth muscle cells

Hui Ding, Quanhua Pan, Long Qian, and Chuanxian Hu*

Department of Cardiovascular Surgery, The Affiliated Huaian No. 1 People’s Hospital of Nanjing Medical University, Huaian, Jiangsu 223300, China

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*Correspondence
Chuanxian Hu
E-mail: huchuanxh@hotmail.com

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ABSTRACT MicroRNAs (miRNAs) regulate gene expression and are biomarkers for coronary atherosclerosis (AS). A novel miRNA-mRNA regulation network of coronary AS still needs to be disclosed. The aim of this study was to analyze potential mRNAs in coronary AS patients and the role of their upstream miR-491-5p in vascular smooth muscle cells (VSMCs). We first confirmed top ten mRNAs according to the analysis from Gene Expression Omnibus database (GSE132651) and examined the expression levels of them in the plaques and serum from AS patients. Five mRNAs (UBE2G2, SLC16A3, POLR2C, PNO1, and AMDHD2) presented significantly abnormal expression in both plaques and serum from AS patients, compared with that in the control groups. Subsequently, they were predicted to be targeted by 11 miRNAs by bioinformatics analysis. Among all the potential upstream miRNAs, only miR-491-5p was abnormally expressed in the plaques and serum from AS patients. Notably, miR-491-5p overexpression inhibited viability and migration, and significantly increased the expression of contractile markers (α-SMA, calponin, SM22α, and smoothelin) in VSMCs. While silencing miR-491-5p promoted viability and migration, and significantly suppressed the expression of α-SMA, calponin, SM22α, and smoothelin. Overall, miR-491-5p targeted UBE2G2, SLC16A3, and PNO1 and regulated the dysfunctions in VSMCs.

INTRODUCTION

Coronary Atherosclerosis (AS) is a multifactorial chronic disease characterized by accumulation of lipids and fibrous components in the aorta [1,2]. In the aortic tunica media, vascular smooth muscle cells (VSMCs) are the main cell type [3,4], and the proliferation and migration of VSMCs in intima are associated with the formation of atherosclerotic plaques [5,6]. Due to the long clinical treatment cycle, poor prognosis, and frequent relapse of coronary AS, it is urgent to explore a novel therapeutic target for Coronary AS from the molecular level.

MicroRNAs (miRNAs) are small non-coding RNA molecules that can regulate gene expression at the post-transcriptional level [7-9]. MiRNAs can directly or indirectly modulate plaque forma-
rotic cerebral infarction through modulation of MMP-9 gene, as reported in another study [18].

In this study, we examined the expression of ten potential mRNAs in plaque tissues and serum from coronary AS patients and identified miR-491-5p as an upstream regulator. We hypothesized that miR-491-5p regulated the dysfunctions of VSMCs, which may contribute to novel research strategies for the coronary AS.

**METHODS**

**Clinical specimens**

This study included 22 coronary AS patients (16 males and 6 females; 9 cases aged ≤ 50; 13 cases aged > 50) with coronary endarterectomy at our hospital. Clinical characteristics of patients with coronary AS were provided in Table 1. The healthy control group included 13 patients (6 males and 7 females; 5 cases aged ≤ 50; 8 cases aged > 50) who underwent physical examinations in the same period at our hospital. The patients with complications or infections of liver or kidney, diabetes, or tumors were excluded. The Gensini scoring system was applied to evaluate the degree of AS. Written informed consent was obtained from all participants. The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of The Affiliated Huaian No. 1 People’s Hospital of Nanjing Medical University (IRB: 2016-045, Jiangsu, China).

Plaque tissues and adjacent intimal tissues were taken by coronary endarterectomy and then stored in liquid nitrogen. Peripheral blood was collected, and serum was separated by centrifugation at 400 g for 10 min.

**Cell culture and transfection**

VSMCs were dissected from perioperative aortic punch samples of participating patients as previous study described [19]. The dissected VSMCs were then cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Gibco fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 µg/ml penicillin-streptomycin (Sigma-Aldrich) at 37°C and 5% CO2. The 10 nM miR-491-5p mimics were used for miR-491-5p overexpression and an equal quantity of miR-491-5p antagonir was used for miR-370 downregulation. Simultaneously, NC mimics and NC antagonir was used in the present study. To downregulate UBE2G2, SLC16A3, and PNO1 expression, short hairpin RNAs targeting UBE2G2, SLC16A3, and PNO1 (sh-UBE2G2, sh-SLC16A3, and sh-PNO1) were used. The miR-491-5p mimic, miR-491-5p antagonir, sh-UBE2G2, sh-SLC16A3, and sh-PNO1, and their relevant controls were all synthesized by GenePharma (Shanghai, China). Cell transfections were conducted for 48 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

**Reverse transcription quantitative polymerase chain reaction**

Total RNA was extracted using TRIzol reagent (Invitrogen). Synthetic spike-in miRNAs (MiRXES, Singapore) was prepared for miRNAs to obtain RNA samples, which was then reverse transcribed into cDNA. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis was performed using mSMRT-qPCR miRNA assay platform (MiRXES, Singapore). The relative expression levels of miRNAs were normalized to U6, and the relative expression levels of mRNAs were normalized to GAPDH, which were all quantified using the 2−ΔΔCq method. Sequences of primers are provided in Table 2.

**Western blot analysis**

Total protein extraction was performed, and the protein concentration was measured. Proteins (25 µg) were then loaded on 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with non-fat milk (5%) at room temperature for 1 h, the membranes were then incubated with the primary antibodies below at 4°C overnight: UBE2G2 (ab74296; 1:1,000; Abcam, Shanghai, China), SLC16A3 (ab244385; 1:1,000; Abcam), POLR2C (ab182150; 1:100; Abcam), PNO1 (ab227978; 1:1,000; Abcam), AMDHD2 (ab133306; 1:1,000; Abcam), α-SMA (ab5831; 1:1,000; Abcam), etc.

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**Table 1. Clinical characteristics of patients with coronary atherosclerosis**

| Parameters                  | Cases (n) |
|-----------------------------|-----------|
| Age                         |           |
| ≤ 50                        | 9         |
| > 50                        | 13        |
| Sex                         |           |
| Female                      | 6         |
| Male                        | 16        |
| Diabetes mellitus           |           |
| Yes                         | 10        |
| No                          | 12        |
| Hypertension                |           |
| Yes                         | 11        |
| No                          | 11        |
| Hyperlipidemia              |           |
| Yes                         | 16        |
| No                          | 6         |
| Smoking                     |           |
| Yes                         | 14        |
| No                          | 8         |
| Ejection fraction           |           |
| Good                        | 12        |
| Fair                        | 8         |
| Poor                        | 2         |

p-values less than 0.05 indicate statistical significance. Chi-square test was performed.
Role of miR-491-5p in vascular smooth muscle cells

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Table 2. Primers used for quantitative RT-PCR

| Name      | Sequence (5'-3') Forward | Sequence (5'-3') Reverse |
|-----------|--------------------------|--------------------------|
| UBE2G2    | GAAGGAATTGTAGCAGGGCC     | CATCAGGGTAGATGTGGGGA    |
| SNORD45C  | GATGAGTTGGGATATTTTGT    | GTGCCTGAGTAATTCTAGAGC   |
| SNORD45A  | GCCGAGGATGTTGGGCCGGA    | GTGCCTGAGTAATTCTAGAGC   |
| SLC16A3   | GAGATCTGCTTCGAGGGC      | GGAATTTTCAGAGATTTGAGGG |
| POLR2C    | CACAGGCTGGATATTTTGT    | ACTCCTCAATGTCAGAGT   |
| PNO1      | GAAGATCTCAGCTTTTCGAGCTC | GGAATTTTCAGAGATTTGAGGG |
| CEMPI     | ACCTCCTAACAATCAGGGG    | TACCTCAATGTCAGAGT   |
| AMDHD2    | GAAGTCTGCTTCGAGGGC     | TACCTCAATGTCAGAGT   |
| miR-491-5p| GAGATCTGCTTCGAGGGC     | TACCTCAATGTCAGAGT   |
| miR-465-5p| GCGACGAGGAAAGATTTTG    | AGAAGATCTCAGCTTTTCGAGCTC |
| miR-4673  | ACCTCCTAGCTTTTCGAGCTC | TCGCGACTCAACTGTGCCC  |
| miR-6791-5p| AGCCGCGCGAGAGA    | GTCTCGCTTCGAGGT   |
| miR-727-3p| GCCTGGCGACCCAGCCGCAGA | CTCGGGGGACGAGGGCTTGG |
| miR-6747-3p| GAGAAGAGCGGAGGACA     | GCCGACGGCGGGAC                 |
| miR-4308  | AGGTAAGAAGAACTCCAAGGAA | AGGTAAGAAGAAGAACTCCAAGGAA    |
| miR-4722-3p| GCCGAGGAGGCTGTGCAAGTTG | AGGTAAGAAGAAGAACTCCAAGGAA    |
| miR-4292  | TTTATACGCTATTACTTGAATTC | TTATACGCTATTACTTGAATTC       |
| miR-6852-5p| CCGATGGGATCTGTACGGAGAT | CATGTCCTCAGACCTCAGGCC    |
| miR-7160-5p| TGCTUGAGGTCTCGGGCTGCGC | TGCTUGAGGTCTCAGGCC    |

Abcam), calponin (ab46794; 1:5,000; Abcam), SM22α (ab14106; 1:1,000; Abcam), smoothelin (ab8969; 1:100; Abcam), GAPDH (as a loading control; ab8245; 1:500; Abcam). Subsequently, the secondary antibody of HRP-conjugated goat anti-rabbit IgG (1:3,000) was added and incubated at room temperature for 1 h. Finally, the protein bands were detected by the ECL Plus Kit (Pierce, Rockford, IL, USA). The relative protein expression was analyzed using ImageJ software 1.4.

RNA pull down assay

Bio miR-491-5p and Bio NC were co-transfected to VSMCs for 2 days. VSMCs (1 × 10^7) were dissolved in the soft lysis buffer plus RNasin (80 U/ml; Promega, Madison, WI, USA). Subsequently, streptavidin agarose beads (Life Technologies, Rockville, MD, USA) were incubated with the reaction mixture for 1 h at room temperature. RT-qPCR was prepared to evaluate the co-precipitated RNAs.

Cell counting kit-8 assay

VSMCs were seeded in 96-well plates (5 × 10^3 cells/well) for 24 h at 37°C and then used to assess cell proliferation. After the transfection, the cell proliferation value was measured by a cell counting kit 8 (CCK-8 kit; Thermo Fisher Scientific, Inc.) at 24, 48, and 72 h. CCK-8 reagent (10 µl) was then added and incubated at 37°C for 1 h. Subsequently, the optical density was measured at 450 nm.

Wound healing assay

VSMCs were cultured in 6-well plates and cultured for 24 h. Subsequently, the parallel lines were scratched on the cell culture plate with a 200 µl pipette tip. Following washing with PBS three times, cells were cultured in serum-free DMEM for an additional 48 h. The wounded gaps were photographed at different time points (0 and 24 h).

Statistical analyses

Data are shown as the mean ± standard deviation. The Student’s t-test was used to assess the differences between two groups. Two-way ANOVA was conducted for difference comparison in groups with two independent variables. All data were statistically analyzed by the SPSS 21.0 (IBM Co., Armonk, NY, USA) statistical software. p-values of less than 0.05 was defined as significant.

RESULTS

Expression of 10 mRNAs in plaques and adjacent intimal tissues from AS patients

According to the gene profiling by array results of GSE132651 which determined differences in blood outgrowth endothelial cells from subjects with normal or abnormal coronary endothelial function, top ten genes were differentially expressed (Table 3). Subsequently, we measured the expression levels of UBE2G2, SNORD45C, SNORD45A, SNORD45B, RABGTTB, SLC16A3,
POLR2C, PNO1, CEMP1, AMDHD2 using RT-qPCR. The levels of UBE2G2, SNORD45C, SNORD45A, SLC16A3, POLR2C, PNO1, AMDHD2 in plaques from coronary AS patients were significantly higher than those in adjacent intima. On the contrary, CEMP1 expression was downregulated in plaques from coronary AS patients, compared with that in adjacent intima. However, the expression levels of SNORD45B and RABGGTB had no significant difference in plaques and adjacent intima from coronary AS patients (Fig. 1A). Only mRNAs (UBE2G2, SNORD45C, SNORD45A, SLC16A3, POLR2C, PNO1, CEMP1, AMDHD2) with significant difference were listed in Fig. 1B.

Expression of 10 mRNAs in serum from AS patients and healthy controls

The levels of UBE2G2, SNORD45B, RABGGTB, SLC16A3, POLR2C, PNO1, and AMDHD2 in the serum of coronary AS patients were significantly higher than those in the serum of healthy patients. While the expression levels of SNORD45C, SNORD45A, and CEMP1 had no significant difference in the serum of coronary AS patients and healthy patients (Fig. 2A). In Fig. 2B, mRNAs (UBE2G2, SNORD45B, RABGGTB, SLC16A3, POLR2C, PNO1, and AMDHD2) with significant difference were listed.

Potential upstream miRNAs for the abnormally expressed mRNAs

We found that five mRNAs (UBE2G2, SLC16A3, POLR2C, PNO1, and AMDHD2) were significantly abnormally expressed in both plaques and sera from subjects with coronary AS. TargetScan was used to predict the potential upstream miRNAs of these five genes, and 11 common miRNAs (hsa-miR-491-5p, hsa-miR-4645-5p, hsa-miR-4673, hsa-miR-6791-5p, hsa-miR-6727-3p, hsa-miR-6747-3p, hsa-miR-4308, hsa-miR-4722-3p, hsa-miR-4292, hsa-miR-6852-5p, and hsa-miR-7160-5p) were identified (Fig. 3A). We then examined the expression levels of these miRNAs using RT-qPCR. Only miR-491-5p showed significantly downregulated expression in plaques from coronary AS patients, compared with that in adjacent intima (Fig. 3B). MiR-491-5p expression was also decreased in the serum of coronary AS patients, compared with that in the sera of healthy patients (Fig. 3C). Collectively, miR-491-5p was a potential upstream miRNA for abnormally expressed UBE2G2, SLC16A3, POLR2C, PNO1, and AMDHD2.

MiR-491-5p negatively regulates UBE2G2, SLC16A3, PNO1 expression in VSMCs

To confirm the predicted regulatory role of miR-491-5p on target genes, we conducted the subsequent experiments in VSMCs separated from the aortic wall sections of coronary AS patients. According to the results of RT-qPCR, miR-491-5p expression was higher in the VSMCs transfected with miR-491-5p mimics than that in the control group (Fig. 4A). mRNA expression and protein levels of UBE2G2, SLC16A3 and PNO1 were significant downregulated in the cells transfected with miR-491-5p mimics, compared with those in the control group, as evaluated by RT-qPCR and western blotting, respectively (Fig. 4B, C). However, the mRNA expression and protein levels of POLR2C and AMDHD2 had no significant difference in the cells transfected with miR-491-5p mimics and NC mimics. Similarly, RNA pull down assay revealed that the expression levels of UBE2G2, SLC16A3 and PNO1 were upregulated in VSMCs transfected with Bio miR-491-5p, compared with those in control groups (Fig. 4D). These results indicated that the expression levels of UBE2G2, SLC16A3 and PNO1 were negatively regulated by miR-491-5p.

MiR-491-5p inhibits viability, migration, and increased expression of contractile markers in VSMCs

The function of miR-491-5p in VSMCs was then explored. CCK-8 revealed that the viability was suppressed in VSMCs transfected with miR-491-5p mimics, compared with that in control group (Fig. 5A). According to the results of the wound healing assay, the migration capacity was decreased in VSMCs

Table 3. Top 10 genes that are differentially expressed in blood outgrowth endothelial cells from subjects with normal or abnormal coronary endothelial function

| Gene symbol | Chromosome location | Gene title | p-value |
|-------------|---------------------|------------|---------|
| UBE2G2      | 21q22.3             | Ubiquitin conjugating enzyme E2 G2 | 0.0000145 |
| SNORD45C    | 1p31.1              | Small nucleolar RNA, C/D box 45C | 0.0000151 |
| SNORD45A    | 1p31.1              | Small nucleolar RNA, C/D box 45A | 0.0000151 |
| SNORD45B    | 1p31.1              | Small nucleolar RNA, C/D box 45B | 0.0000151 |
| RABGGTB     | 1p31                | Rab geranylgeranyltransferase beta subunit | 0.0000151 |
| SLC16A3     | 17q25               | Solute carrier family 16 member 3 | 0.0000213 |
| POLR2C      | 16p13-q21           | RNA polymerase II subunit C | 0.0000223 |
| PNO1        | 2p14                | Partner of NOB1 homolog | 0.0000265 |
| CEMP1       | 16p13.3             | CEMENTUM protein 1 | 0.0000299 |
| AMDHD2      | 16p13.3             | Amidohydrolase domain containing 2 | 0.0000299 |

Data were downloaded from GSE132651.
transfected with miR-491-5p mimics, compared with that in control group (Fig. 5B). Importantly, the protein levels of contractile markers in VSMCs were elevated by miR-491-5p mimics (Fig. 5C). These results suggested that miR-491-5p inhibited viability, migration, and increased expression of contractile markers in VSMCs.

**MiR-491-5p antagomir promotes viability, migration, and increased expression of contractile markers in VSMCs**

To further confirm the function of miR-491-5p and its target genes UBE2G2, SLC16A3, and PNO1 in VSMCs, miR-491-5p
antagomir, sh-UBE2G2, sh-SLC16A3, and sh-PNO1 were transfected in VSMCs and NC antagomir and sh-NC were used as negative control. A CCK-8 assay revealed that silencing miR-491-5p in VSMCs significantly promoted cell proliferation when compared with the control group at 24, 48, and 72 h, while UBE2G2, SLC16A3, and PNO1 knockdown partially reversed the changes (Fig. 6A). Wound healing assay was performed to assess cell migration. The results indicated that the inhibition of miR-491-5p significantly upregulated VSMCs migration. However, the upregulation was then alleviated by UBE2G2, SLC16A3, and PNO1

Fig. 2. Expression of 10 mRNAs in serum from atherosclerosis (AS) patients and healthy controls. (A) Expression levels of ten mRNAs (UBE2G2, SNORD45C, SNORD45A, SNORD45B, RABGGTB, SLC16A3, POLR2C, PNO1, CEMP1, AMDHD2) in the serum of coronary AS patients and healthy patients were measured using RT-qPCR with unpaired t-test. (B) mRNAs (UBE2G2, SNORD45C, SNORD45A, SLC16A3, POLR2C, PNO1, CEMP1, AMDHD2) with significant difference were listed. *p < 0.05, **p < 0.01, ***p < 0.001, NS indicates no significance.
knockdown (Fig. 6B). Moreover, the protein levels of α-SMA, calponin, SM22α, and smoothelin were suppressed by miR-491-5p antagonir, While UBE2G2, SLC16A3, and PNO1 knockdown partially restored the changes, as shown by western blotting (Fig. 6C). Taken together, these results suggested that miR-491-5p silencing promotes VSMCs proliferation and migration but inhibits the expression of contractile markers. However, UBE2G2, SLC16A3, and PNO1 knockdown reversed the changes induced by miR-491-5p silencing.

**DISCUSSION**

In our study, we first confirmed ten potential mRNAs that were differentially expressed in blood outgrowth endothelial cells from subjects with normal or abnormal coronary endothelial function, according to the gene profiling by array results of GSE132651. After evaluating their expression levels, we found that expression of five mRNAs (UBE2G2, SLC16A3, POLR2C, PNO1, and AMDHD2) had significant difference in both plaques and serum from...
Fig. 4. MiR-491-5p negatively regulates UBE2G2, SLC16A3, PNO1 expression in vascular smooth muscle cells (VSMCs). (A) Overexpression efficiency of miR-491-5p in VSMCs was evaluated using RT-qPCR. (B) Expression levels of UBE2G2, SLC16A3, POLR2C, PNO1, and AMDHD2 in VSMCs transfected with miR-491-5p mimics and NC mimics were examined by RT-qPCR. (C) Protein levels of UBE2G2, SLC16A3, POLR2C, PNO1, and AMDHD2 in VSMCs transfected with miR-491-5p mimics and NC mimics were examined by Western blot. (D) RNA pull down assay was for detecting whether UBE2G2, SLC16A3, and PNO1 bind with miR-491-5p. *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 5. MiR-491-5p inhibited viability, migration, and increased expression of contractile markers in vascular smooth muscle cells (VSMCs). (A) Cell counting kit 8 (CCK-8) revealed the viability in VSMCs with transfection of miR-491-5p mimics and NC mimics. (B) Wound healing was performed to detect the migration capacity of VSMCs with transfection of miR-491-5p mimics and NC mimics. (C) The protein levels of contractile markers (α-SMA, calponin, SM22α, and smoothelin) in VSMCs with transfection of miR-491-5p mimics and NC mimics were measured by Western blot. *p < 0.05, **p < 0.01.
Fig. 6. MiR-491-5p antagomir promotes viability, migration, and increased expression of contractile markers in vascular smooth muscle cells (VSMCs). (A) CCK-8 revealed the viability in VSMCs with transfection of NC antagomir, miR-491-5p antagomir, miR-491-5p antagomir+sh-NC, miR-491-5p antagomir+sh-UBE2G2, miR-491-5p antagomir+sh-SLC16A3, and miR-491-5p antagomir+sh PNO1. (B) Wound healing was performed to detect the migration capacity of VSMCs with transfection of NC antagomir, miR-491-5p antagomir, miR-491-5p antagomir+sh-NC, miR-491-5p antagomir+sh-UBE2G2, miR-491-5p antagomir+sh-SLC16A3, and miR-491-5p antagomir+sh PNO1. (C) The protein levels of contractile markers (α-SMA, calponin, SM22α, and smoothelin) in VSMCs with transfection of NC antagomir, miR-491-5p antagomir, miR-491-5p antagomir+sh-NC, miR-491-5p antagomir+sh-UBE2G2, miR-491-5p antagomir+sh-SLC16A3, and miR-491-5p antagomir+sh PNO1. *p < 0.05, **p < 0.01.
miR-491-5p significantly promoted VSMCs viability and migration. Molecular mechanisms underlying the miRNA-controlled VSMCs differentiation and coronary AS mainly through the modulation of target gene expression [3,25,26]. Previous studies have reported several miRNA-mRNA regulatory networks in AS [27-29]. For instance, upregulated miR-93 contributes to coronary AS pathogenesis through targeting ABCA1 [30]. MiR-126 represses the progression of coronary AS in the mice by binding to SIPR2 [31]. MiR-491-5p has been once explored in VSMCs in a previous study, which demonstrates its inhibitory role on the growth and migration of VSMCs by binding with MMP-9 gene, indicating the involvement of miR-491-5p in AS development [17]. Particularly, we found three mRNAs UBE2G2, SLC16A3, and PNO1 that were negatively regulated by miR-491-5p, which were not mentioned in any previous research. Mechanically, UBE2G2, SLC16A3, and PNO1 were validated as the targets downstream miR-491-5p, indicating the upregulation of UBE2G2, SLC16A3, and PNO1 in plaques and serum from AS patients was induced by the down-regulation of miR-491-5p. Furthermore, the functions of miR-491-5p, UBE2G2, SLC16A3, and PNO1 in smooth muscle cells were assessed. Notably, we found that miR-491-5p overexpression inhibited viability, migration, and increased the expression of contractile markers α-SMA, calponin, SM22α, and smoothelin in VSMCs. The increase of these contraction markers suggests that miR-491-5p overexpression promoted the differentiation of phenotypic proteins, which is a key element in AS development because the synthetic phenotype of VSMCs can migrate, proliferate, and generate extracellular matrix proteins [5]. Inversely, silencing miR-491-5p significantly promoted VSMCs viability and migration, and inhibited the expression of α-SMA, calponin, SM22α, and smoothelin in VSMCs. However, UBE2G2, SLC16A3, and PNO1 knockdown reversed the changes induced by miR-491-5p antagonir.

In conclusion, this study revealed the differentially expressed mRNAs and their upstream miR-491-5p in patients with coronary atherosclerosis. We also found that miR-491-5p inhibited viability, migration, and increased expression of contractile markers via regulating target genes UBE2G2, SLC16A3, and PNO1 in VSMCs. These results might provide a novel insight into the molecular mechanisms underlying the miRNA-controlled VSMCs dysfunctions in diagnostic and therapeutic approaches against coronary AS.

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