Original Article

miR-29a regulates VSMC cell proliferation and its roles in the pathogenesis of hypertension through targeting PTEN

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Abstract: Vascular smooth muscle cell (VSMC) hyperplasia is related to hypertension. Phosphatase and tensin homology deleted on chromosome ten (PTEN) is the inhibitor of PI3K/AKT signaling pathways. Bioinformatics analysis has demonstrated the targeting relationship between miR-29a and PTEN mRNA 3’-UTR. The current study investigated the roles of miR-29a in regulating PTEN expression, VSMC proliferation, and the pathogenesis of hypertension. miR-29a, PTEN, p-AKT, and Ki67 expression in the media of SHR and WKY rats was compared. The targeted relationship between miR-29a and PTEN was confirmed by dual luciferase reporter assay. SHR rats were randomly divided into two groups, antagomir-29a and antagomir-NC groups. Systolic blood pressure (SBP) and diastolic pressure (DBP) of the caudal arteries were compared. PTEN and p-AKT expression in the blood vessels was detected by Western blot. VSMCs were cultured in vitro and divided into two transfection groups, the miR-NC group and miR-29a inhibitor group. Cell proliferation was tested by EdU staining. Compared with WKY rats, miR-29a, p-AKT, and Ki67 expression was significantly increased, while PTEN levels were obviously declined in the vascular media of SHR rats. In vivo injections of antagomir-29a upregulated PTEN expression, reduced p-AKT and Ki67 levels, and decreased systolic and diastolic blood pressure in the vascular tissue of rats. Transfection of miR-29a markedly enhanced PTEN expression, decreased p-AKT expression, and weakened cell proliferation in VSMC cells in vitro. miR-29a plays a role in lowering blood pressure by inhibiting PTEN, enhancing the activity of PI3K/AKT signaling pathways, and suppressing VSMC cell proliferation.

Keywords: Hypertension, miR-29a, PTEN, PI3K/AKT, VSMC

Introduction

Hypertension is a common cardiovascular disease characterized by an abnormal increase in systemic blood pressure [1-3]. Under pathological conditions, excessive proliferation of VSMC can migrate to the intima, narrow the vascular lumen, and thicken blood vessel walls. Therefore, it cannot effectively regulate the physiological fluctuations of blood pressure, resulting in enhanced vascular resistance and hypertension [4].

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT/PKB) is an important signaling pathway widely expressed in various tissues and cells. Excessive activation of PI3K/AKT signaling pathways is closely related to elevated blood pressure [5, 6]. PTEN is an inhibitor of PI3K/AKT signaling pathways [7]. Its expression and functional activity reduction plays a regulatory role in pulmonary arterial hypertension (PAH) [8-11]. However, the relationship between PTEN and hypertension remains unclear. MicroRNAs are newly discovered non-coding single-stranded small molecule RNAs at length of 19 to 25 nucleotides. They are extremely important gene regulatory substances, playing a key role in the regulation of about 30% of gene transcription and expression [12, 13]. MicroRNAs mainly participate in cell growth, differentiation, apoptosis, and migration, through antisense complementary binding with the 3’-untranslated region (3’-UTR) of the target gene mRNA. This leads to target mRNA degradation or inhibition at post-transcriptional levels [14, 15]. It has been shown that abnormally elevated miR-29a expression is associated with
development of hypertension [16, 17]. Bioinformatics analysis has revealed a complementary binding site between miR-29a and the 3'-UTR of PTEN mRNA, suggesting a potential targeted regulatory relationship. The current study compared expression of miR-29a and PTEN in the vascular media of hypertensive rats, exploring the roles of miR-29a in regulating PTEN expression, affecting the activity of PI3K/AKT signaling pathways and the pathogenesis of hypertension.

**Materials and methods**

**Main reagents and materials**

Male SPF spontaneous hypertensive rats (SHR) and male Wistar-Kyoto rats (WKY), 10 weeks old and weighing 120-150 g, were purchased from the Yangzhou University Medical Experimental Animal Center (Yangzhou, Jiangsu, China). DMEM medium was purchased from Biological Industries. Fetal bovine serum (FBS), Opti-MEM medium, type II collagenase, Lip2000, and TRIzol were purchased from Thermo. Antagomir-29a, antagomir-NC, miR-29a mimics, miR-29a inhibitors, miR-NC, and EdU-stained cell proliferation flow detection kits were purchased from Ribobio. Rabbit anti-PTEN, AKT, p-AKT, and β-actin polyclonal antibodies were purchased from Abcam. HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody was purchased from Sangon. Flow cytometry antibody PE-Ki67 was purchased from Biolegend. Annexin V/PI apoptosis detection kit was purchased from Beyotime. Luciferase activity detection reagent and pGL3 plasmid were purchased from Promega. PrimeScript™ RT kit was purchased from Takara.

**SHR rat grouping and treatment**

A total of 10 SHR rats were randomly divided into the antagomir-29a group and antagomir-NC group. The rats received caudal vein injections at 10 mg/Kg once per three days for five times. Caudal artery systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured before and on the 7th day after the last injection.

**Rat thoracic aorta VSMC isolation and cultivation**

Rats were anesthetized by pentobarbital tail vein injections. The thoracic aorta was extract-
After the termination of the reaction by adding 100 μL of Stop&Glo. The ratio of the fluorescence value I/fluorescence value II was the relative luciferase activity.

**VSMC cell transfection**

VSMCs derived from SHR rats were cultured in vitro and divided into three groups, the control group, miR-NC group, and miR-29a inhibitor group. miR-NC, miR-29a inhibitors, and Lip-2000 were diluted by Opti-MEM and incubated at room temperature for 5 minutes. Next, miR-NC and miR-29a inhibitors were mixed with Lip2000 and incubated for 30 minutes at room temperature. The VSMC cell culture medium was replaced with Opti-MEM serum-free cell culture medium. The transfection complex was added to the culture medium and incubated for 6 hours. It was then replaced with DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. After 72 hours, the cells were collected by trypsin digestion for the following experiments.

**qRT-PCR**

Total RNA was extracted using TRlzol and reverse transcribed to cDNA using the PrimeScript™ RT reagent Kit. The PCR reaction system contained 5.0 μl 2×QuantiTect SYBR Green Mixture, 0.5 μl primer at 5 μm/L, 1 μl Template RNA, and ddH₂O. RT conditions were 50°C for 15 minutes and 85°C for 5 minutes. PCR reactions were composed of 95°C pre-denaturation for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Real-time PCR was performed on CFX96 to test relative expression levels.

**Western blotting**

Total protein was extracted by RIPA on ice for 15 minutes and centrifuged at 4°C and 10000 g for 10 minutes. The supernatant was moved to a new Ep tube for quantification. A total of 40 μg protein was separated by 10% SDS-PAGE for 3 hours, then transferred to PVDF membranes at 300 mA for 90 minutes. Next, the membrane was blocked in 5% skim milk at room temperature for 60 minutes and incubated in the primary antibody at 4°C overnight (PTEN, AKT, p-AKT, and β-actin at 1:2000, 1:1000, 1:2000, and 1:10000, respectively). The membrane was incubated in a secondary antibody (1:15000) for 60 minutes after washing with PBST three times. Lastly, protein expression was detected by ECL chemiluminescence.

**Cell apoptosis detection**

The cells were collected and added with 100 μL binding buffer. Next, the cells were incubated in 5 μL Annexin V-FITC and 5 μL PI, void of light, for 10 minutes. The cells were then resuspended after adding 400 μL binding buffer. Cell apoptosis was tested using Gallios flow cytometry (Beckman Coulter).

**EdU staining**

VSMC cells were seeded in 6-well plates and added with EdU solution at 10 μM in the logarithmic phase. After incubation for 48 hours, the cells were digested by trypsin and collected. After being fixed in 4% paraformaldehyde, the cells were incubated in 0.5% TritonX-100 at room temperature for 15 minutes and in 500 μL reaction fluid at room temperature, void of light, for 30 minutes. Lastly, the cells were tested using flow cytometry.

**Statistical analysis**

Data analyses were performed using SPSS 18.0 software. Measurement data are depicted as mean ± standard deviation and compared by t-test. P < 0.05 indicates statistical significance.

**Results**

MicroRNA.org online target gene prediction showed the complementary binding site between miR-29a and the 3'-UTR of PTEN mRNA (Figure 1A). Dual luciferase assay revealed that miR-29a mimics or miR-29a inhibitors significantly declined or elevated the relative luciferase activity in VSMC cells transfected by pGL3-PTEN-wt but not pGL3-PTEN-mut (Figure 2B), indicating the regulatory relationship between miR-29a and PTEN mRNA.

miR-29a upregulated, while PTEN declined, in the vascular media tissue of SHR rats

qRT-PCR showed that expression of miR-29a was significantly increased, while expression of PTEN mRNA was obviously reduced in the vascular media tissue of SHR rats, compared with WKY rats (Figure 2A). Western blotting exhibit-
Downregulation of miR-29a increased PTEN expression and reduced blood pressure in SHR rats

qRT-PCR showed that, compared with the antagonist-NC group, expression of miR-29a in vivo was significantly reduced, while expression of PTEN mRNA was obviously upregulated in the vascular media tissue of SHR rats injected with antagonist-29a (Figure 3A). Western blotting demonstrated that, compared with the antagonist-NC group, expression of PTEN proteins was markedly enhanced and expression of p-AKT proteins was apparently declined in the vascular media tissue of SHR rats injected with antagonist-29a (Figure 3B). Flow cytometry revealed that in vivo injections of antagonist-29a significantly attenuated expression of Ki67 in the vascular media tissue of SHR rats (Figure 3C). Blood pressure measurements revealed that, compared with the antagonist-NC group, SBP (Figure 3D) and DBP (Figure 3E) were significantly decreased in the antagonist-29a group.

Discussion

Genetics, high-salt diets, mental stress, age, and drugs are risk factors for onset of hypertension [1-3]. Due to improvements in living standards, changes in dietary structures, accelerated pace of life, increased work pressures, irregularities in work and rest, and the aging of society, incidence of hypertension is increasing [18]. Under pathological conditions, excessive proliferation of VSMC can migrate to the intima, narrow the lumen, and thicken blood vessel walls. It cannot effectively regulate the physiological fluctuations of blood pressure, resulting in enhanced vascular resistance and hypertension [4]. Therefore, inhibiting the excessive proliferation of VSMC cells may be a strategy that reduces hypertension.

PI3K/AKT is an important signaling pathway widely expressed in many tissues and cells. When PI3K/AKT pathways are activated, P13K can catalyze phosphatidylinositol 4,5-trisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3), which directly phosphorylates and activates AKT to further activate downstream signaling molecules, upregulate cell survival, and promote expression of proliferation and cell cycle progression factors. Thus, it plays a crucial role in regulating various biological processes, such as cell growth, survival, proliferation, and apoptosis. Several studies have shown that excessive activation of PI3K/AKT signaling pathways is closely related to elevated blood pressure [5, 6]. PTEN is the only tumor suppressor gene discovered to have dual
miR-29a in VSMC proliferation and hypertension

Figure 2. miR-29a upregulated, while PTEN declined, in the vascular media tissue of SHR rat. A. qRT-PCR detection of miR-29a and PTEN mRNA expression; B. Western blot detection of protein expression; C. Flow cytometry detection of Ki67 expression. *P < 0.05, compared with WKY.

activities of protein esterase and phosphatase. PTEN can dephosphorylate its substrate PIP3 and inhibit PI3K to block PI3K/AKT signaling pathways [7]. Several studies have found that [8-11] PTEN expression and functional decline play regulatory roles in the pathogenesis of PAH, but the relationship between PTEN and hypertension has been less studied. It has been revealed that abnormally elevated miR-29a expression is associated with the development of hypertension [16, 17]. The current study compared expression of miR-29a and PTEN in the vascular media of hypertensive rats, exploring the roles of miR-29a in regulating PTEN expression, affecting the activity of PI3K/AKT signaling pathways and the pathogenesis of hypertension.

Flow cytometry showed that expression of Ki67 in the vascular media tissue of SHR rats was significantly higher than that of WKY rats, indicating that cell proliferation in the blood vessels of the hypertensive rat model was significantly enhanced. Results of gene and protein expression tests revealed that expression of miR-29a was obviously upregulated, while expression of PTEN was significantly decreased. Expression of p-AKT proteins was significantly increased in the vascular media tissue of SHR rats, compared to WKY rats with normal blood pressure, suggesting that increased expression of miR-29a may play a role in inhibiting PTEN, enhancing the activity of PI3K/AKT pathways, and promoting cell proliferation and the pathogenesis of hypertension. Ravi et al. [11] found that expression of PTEN was significantly reduced and expression of p-AKT was markedly enhanced in arterial VSMC cells of PAH model rats. Horita et al. [10] showed that expression of PTEN was apparently reduced in VSMCs induced by hypoxia, compared with normal controls. The decrease in PTEN expression was associated with enhanced proliferation of VSMC cells. Downregulation of PTEN apparent-
miR-29a in VSMC proliferation and hypertension

Figure 3. Downregulation of miR-29a increased PTEN expression and reduced blood pressure in SHR rat. A. qRT-PCR detection of miR-29a and PTEN mRNA expression; B. Western blot detection of protein expression; C. Flow cytometry detection of Ki67 expression; D. SBP measurement; E. DBP measurement. *P < 0.05, compared with antagomir-NC group.

ly promoted the occurrence and progression of rat pulmonary hypertension. Liu et al. [19] observed that expression of PTEN in pulmonary artery smooth muscle cells (PASMCs) of PAH patients was significantly lower than that of healthy controls and expression of PTEN was associated with hypoxia-induced PASMCs proliferation enhancement. Green et al. [20] showed that expression of PTEN in lung tissues of a hypoxia-induced PAH mouse model was significantly reduced. It was also significantly decreased in hypoxic-treated human PASMCs. Lai et al. [21] found that expression of PTEN in lung tissues of PAH mice was significantly
miR-29a in VSMC proliferation and hypertension

Figure 4. Inhibition of miR-29a upregulated PTEN expression and attenuated VSMC cell proliferation. A. qRT-PCR detection of miR-29a and PTEN mRNA expression; B. Western blot detection of protein expression; C. Flow cytometry detection of VSMC cell proliferation. *P < 0.05, compared with miR-NC group.

Reduced. In the systemic circulation hypertension model, expression of PTEN in vascular media of SHR rats was obviously lower than that of WKY rats, in accord with Green [20], Lai [21], and Horita [10].

Huang et al. [16] confirmed that, compared with the healthy control group, plasma miR-29a expression was significantly increased in hypertensive patients. Khalilq et al. [17] showed that expression of miR-29a in peripheral blood serum of pregnant women with hypertension was markedly higher than that of normal pregnant women. Xu et al. [22] revealed that, compared with hypertensive patients without arterial dissection, expression of miR-29a in peripheral blood of hypertensive patients with arterial dissection was significantly enhanced, indicating that miR-29a upregulation may aggravate hypertension to induce arterial dissection. In this study, expression of miR-29a in the vascular media of hypertensive rats was significantly higher than in the control group, suggesting that elevated miR-29a may be involved in the regulation of hypertension. Present results were consistent with Huang [16] and Khalilq [17].

Dual-luciferase reporter assay showed that transfection of miR-29a mimics or miR-29a inhibitors significantly decreased or increased relative luciferase activity in VSMCs, respectively, indicating that there is a target regulatory relationship between miR-29a and PTEN mRNA. In hypertensive rats, this study observed that in vivo injections of antagomir-29a inhibi-
miR-29a in VSMC proliferation and hypertension

Expression of miR-29a by upregulating expression of PTEN, attenuating the activity of PI3K/AKT pathways, and inhibiting the proliferation of intravascular cells, and lowering blood pressure in rats. In vitro studies also demonstrated that transfection of miR-29a inhibitors can enhance expression of PTEN in VSMC cells, thereby weakening the activity of PI3K/AKT pathways and inhibiting VSMC cell proliferation. Lai et al. [21] found that downregulation of PTEN by siRNA can significantly enhance the proliferation of VSMCs in pulmonary arteries. Prostaglandin E1 (PGE1) treatment can restrain the proliferation of pulmonary VSMCs by upregulating expression of PTEN, indicating that decreased expression of PTEN is a contributing factor to the enhanced proliferation of VSMC cells. This study combined the targeted relationship between miR-29a and PTEN, revealing that miR-29a regulates expression of PTEN, influences the activity of PI3K/AKT pathways, regulates VSMC cell proliferation, and plays a role in the pathogenesis of hypertension. It was observed that PI3K/AKT pathways can regulate cell proliferation, apoptosis, and other biological processes by affecting expression of various downstream genes, such as Bcl-2 [23, 24] and survivin [25]. However, this study did not elucidate specific targeted genes regulated by miR-29a and PTEN-PI3K/AKT pathways.

Conclusion

In the current study, increased expression of miR-29a and decreased expression of PTEN was associated with onset of hypertension. miR-29a plays a role in lowering blood pressure by inhibiting PTEN, enhancing the activity of PI3K/AKT signaling pathways, and suppressing VSMC cell proliferation.

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Disclosure of conflict of interest

None.

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miR-29a in VSMC proliferation and hypertension


