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Original Article

Regulation of microRNA-214 on vascular smooth muscle cell proliferation and potential treatment effects in hypertension mouse

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Abstract: The over-proliferation of vascular smooth muscle cell (VSMC) is closely correlated with hypertension. Phosphatase and tensin homology deleted on chromosome ten (PTEN) gene negatively regulates PI3K/AKT signal pathway, inhibits VSMC proliferation and facilitates its apoptosis. Lower PTEN and higher microRNA (miR)-214 expression are all related with pulmonary artery hypertension, but leaving the correlation with hypertension unknown. Bioinformatics analysis revealed targeted relationship between miR-214 and 3'-UTR of PTEN mRNA. This study thus established if miR-214 played a role in regulating PTEN expression, VSMC proliferation, apoptosis and hypertension. Expression of miR-214, PTEN and p-AKT was compared between SHR and WKY rat vascular membrane. Dual luciferase reporter gene assay confirmed targeted regulation between miR-214 and PTEN. *In vitro* cultured VSMC was treated with miR-214 inhibitor and/or pIRES2. Flow cytometry was used to measure cell apoptosis and Ki67 expression. Western blot was used for detecting PTEN and p-AKT expression. SHR rats received antagomir-214 for measuring tail artery systolic/diabolic pressure. PTEN expression was measured by Western blot. SHR rats had elevated miR-214 and p-AKT in vascular membrane compared to WKY rats, along with lower PTEN expression. MiR-214 targted and inhibited PTEN expression. MiR-214 inhibitor transfection and/or PTEN overexpression decreased p-AKT expression and cell proliferation, and enhanced apoptosis. *In vivo* injection of antagomir-214 remarkably decreased blood pressure or PTEN expression. MiR-214 targeted and inhibited PTEN expression, enhanced PI3K/AKT activity, facilitated VSMC proliferation, and decreased apoptosis. Antagomir-214 exerts anti-hypertension via decreasing PTEN expression.

Keywords: MicroRNA-214, PTEN, VSMC, hypertension, cell proliferation, cell apoptosis

Introduction

Hypertension is one common cardiovascular disease that easily leads to coronary heart disease, cardiomyocyte fibrosis, cardiac hypertrophy, heart failure, cerebral stroke and other cardio-cerebro-vascular disease, thus severely affecting human health and life [1, 2]. Current opinions believed that hypertension was one type of diseases featured with abnormal hyperplasia of vascular smooth muscle cell (VSMC), whose over-proliferation and downward migration toward the inner membrane play a crucial role in constricting vascular cavity, thickening vascular wall, increasing peripheral vascular resistance and blood pressure [3]. Phosphatase and tensin homology deleted on chromosome ten (PTEN) gene discovered in 1997 was the only one tumor suppressor gene with dual proteinase and phosphatase activity ever been found, and is widely involved in the regulation of cell proliferation, apoptosis, migration and differentiation [4]. Both *in vivo* [5] and *in vitro* [6] studies all demonstrated that PTEN could inhibit the proliferation of VSMC and facilitated their apoptosis via negative regulating PI3K/AKT signal pathway which is crucial for cell proliferation and survival. Various studies showed the possible correlation between PTEN expression/functional deficits and pathogenesis of pulmonary artery hypertension. Its role in hypertension, however, is still unclear [7, 8]. MicroRNA is one family of non-coding small molecule single stranded RNA with 18~22 nucleotides length, and is one important factor in epigenetics regulation. It can bind onto 3'-UTR of target gene

mRNA via complete or incomplete complementary binding manner, in order to degrade mRNA or inhibit gene translation. It can regulate more than one third of human gene expression, and participates in various biological processes including cell proliferation, apoptosis and migration. Increasing evidences showed that abnormal expression and function of miR plays a role in hypertension occurrence [9-11]. Previous studies showed the correlation between miR-214 abnormal expression and pathogenesis of pulmonary artery hypertension [12, 13], but leaving its relationship with hypertension largely unclear. Bioinformatics analysis revealed the existence of complementary binding sites between miR-214 and 3'-UTR of PTEN mRNA. This study thus investigated if miR-214 played a role in regulating PTEN expression and affecting VSMC proliferation, apoptosis and hypertension occurrence.

Materials and methods

Reagents and materials

DMEM culture medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (US). Lipofectamine 2000 liposome transfection reagent was purchased from Invitrogen (US). Type II collagenase was purchased from Sigma (US). ReverTra Ace qPCR RT Kit and SYBR Green dye were purchased from Toyobo (Japan). micr*OFF*™ antagnomir-214, micr*OFF*™ antagnomir-control, miR-214 mimic, miR-214 inhibitor and negative control were all synthesized by Ruibo Bio (China). Mouse anti-PTEN antibody was purchased from Abcam (US). anti-rabbit p-AKT antibody was purchased from CST (US). Horseradish peroxidase (HRP) labelled goat anti-rabbit and rabbit anti-mouse secondary antibody were all purchased from Jackson ImmunoResearch (US). FITC labelled Ki-67 was purchased from BD Pharmigen (US). Annexin V/PI apoptotic kit was purchased from Yusheng (China). Dual-Luciferase Reporter assay system and pGL3-promoter were purchased from Promega (US).

Experimental animal

Male SPF grade spontaneous hypertensive rats (SHR) and male normal controlled Wistar-Kyoto rats (WKY) were purchased from Central South University (Hunan, China). Body weight of all rats maintain at 220±20 g while rat age was between 10~12 weeks.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Xiangya Hospital, Central South University.

SHR grouping and treatment

20 SHR rats were randomly divided into two groups (N=10 each): antagomir-214 group, which received 10 mg/kg of micr*OFF*™ antagnomir-214 via tail vein injection every 3 days for 5 times; and antagomir-control group, which received 10 mg/kg of micr*OFF*™ antagnomir-control via tail vein injection every 3 days for 5 times. 7 days before the first injection and 7 days after the last injection, Softron BP-98A was used to measure tail artery systolic blood pressure (SBP) and diabolic blood pressure (DBP) of rats under static state. Average values were obtained from three consecutive measurements of blood pressure.

Separation and culture of VSMC from rat pulmonary aorta

Rats were anesthetized by 10% chloral hydrate and were immersed in 75% ethanol. Under sterile condition, pulmonary aorta was quickly extracted to remove peripheral lipid and mesenchymal tissues around the vessel. 0.1% type II collagenase was used for 30 min digestion at 37°C, followed by the removal of out membrane. Aorta was then longitudinally dissected. Ophthalmology forceps were used to remove inner membrane, leaving middle membrane. Partial tissues were used to extract RNA using Trizol method, partial for protein extraction, and partial tissues were used for VSMC culture.

Middle membrane was cut into 1 mm^3 cubes, and were digested in 0.1% type II collagenase at 37°C for 2 h, and were then digested in 0.05% trypsin for 20 min further. The digestion was stopped by serum. Lysate was then filtered and transferred into 50 mL centrifuge tube, and were centrifuged at 1200 rpm for 5 min. After discarding the supernatant, cells were resuspended into DMEM medium containing 20% FBS and 1% penicillin-streptomycin, and were cultured in 37°C incubator with 5% CO_2. Culture medium was changed after 48 h. Experiments were performed at 4^{th} to 5^{th} generation.

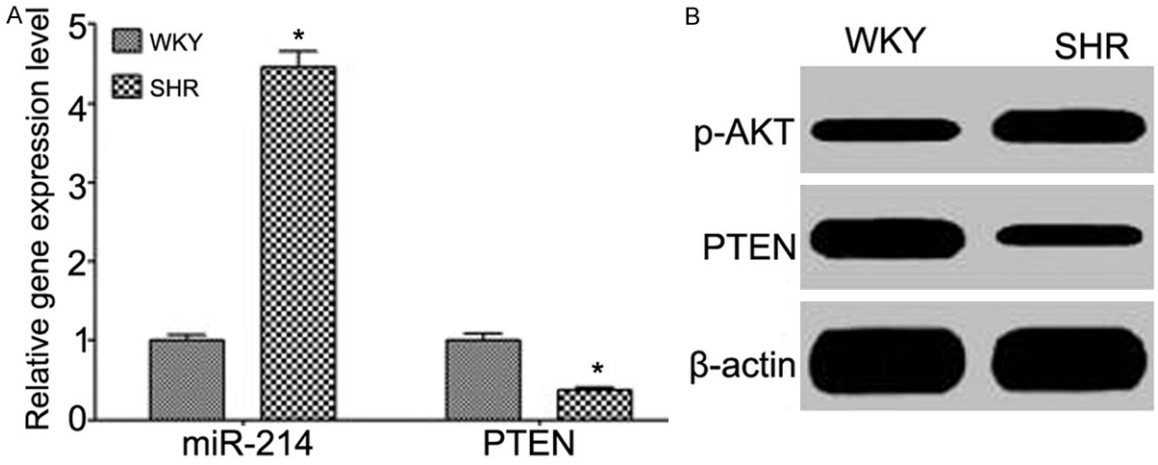

Figure 1. MiR-214 and PTEN expressions in SHR rat vascular middle membrane tissues. A: qRT-PCR for miR-214 and PTEN mRNA expression; B: Western blot for RASSF5 protein expression. *, P<0.05 compared to WKY rats.

Construction of luciferase reporter gene

Using HEK293 genome as the template, full length fragment of 3'-UTR of PTEN gene was amplified. PCR products were purified from agarose gel, and were ligated into pGL-3M luciferase reporter plasmid after XbaI/NotI dual digestion. Recombinant plasmid was then used to transform DH5α competent cells. Positive clones with primary screening were selected for further cell transfection and following experiments.

Luciferase reporter gene assay

Lipofectamine 2000 was used to transfect HEK293 cells with 500 ng pGL3-PTEN-3'UTR plasmid, 30 nmol miR-214 oligonucleotide fragments (or negative control), and 30 ng pRL-TK. After 6 h transfection, normal DMEM medium containing 10% FBS and 1% streptomycin-penicillin was used. After 48 h continuous incubation, dual-luciferase assay was performed. In brief, cells were washed twice in PBS, with the addition of 100 μL PLB lysis buffer. With vortex at room temperature for 30 min, the mixture was centrifuged at 1000 rpm for 10 min. 20 μL cell lysate was mixed with 100 μL LAR II. Fluorescent value I was measured in a microplate reader. The enzymatic reaction was stopped in 100 μL Stop & Glo, followed by quantification of fluorescent value II. The relative expression level of reporter gene was calculated as the ratio of fluorescent value I/fluorescent value II. Oligonucleotide sequences used were: mimic NC, 5'-UUCUC CGAAC GUGUC ACGUU U; miR-214 mimic, 5'-UUGUG CUUGA UCUAA CCAUG UAUGG UUAGA UCAAG CACAA UU; inhibitor NC, 5'-UUCUC CGAAC GUGUC ACGUU U-3'; miR-214 inhibitor, 5'-UUGUG CUUGA UCUAA CCAUG UAUGG UUAGA UCAAG CACAA UU-3'.

Overexpression plasmid construction and cell transfection

Using pIRES as eukaryotic expression plasmid, and Xho I and BamH I as restriction digestion enzyme, amplification primer of PTEN was synthesized using primer 6.0 based on mRNA sequence of PTEN in Gene Bank (Forward, 5'-CAGAC ATGAC AGCCA TCATC A-3'; Reverse, 5'-ATTCA GACTT TTGTA ATTTG TG-3'). PTEN gene was amplified based on cDNA template. Agarose gel electrophoresis was used to determine targeted fragments, which were extracted by gel extraction kit. After ligated with vector, recombinant plasmid was used to transfect competent cell JM109. Ampicillin-containing culture dish was used to collect positive bacterial strain, which was further amplified and extracted for recombinant plasmid containing targeted fragment. Gene sequencing was performed to confirm the correct insertion of target sequence into the plasmid. Lipofectamine 2000 was used to transfect inhibitor NC, miR-214 inhibitor, non-sense controlled plasmid (pIRES2-Scramble), or over-expression plasmid (pIRES2-PTEN) was transfected into VSMC in five groups: inhibitor NC, miR-214 inhibitor,

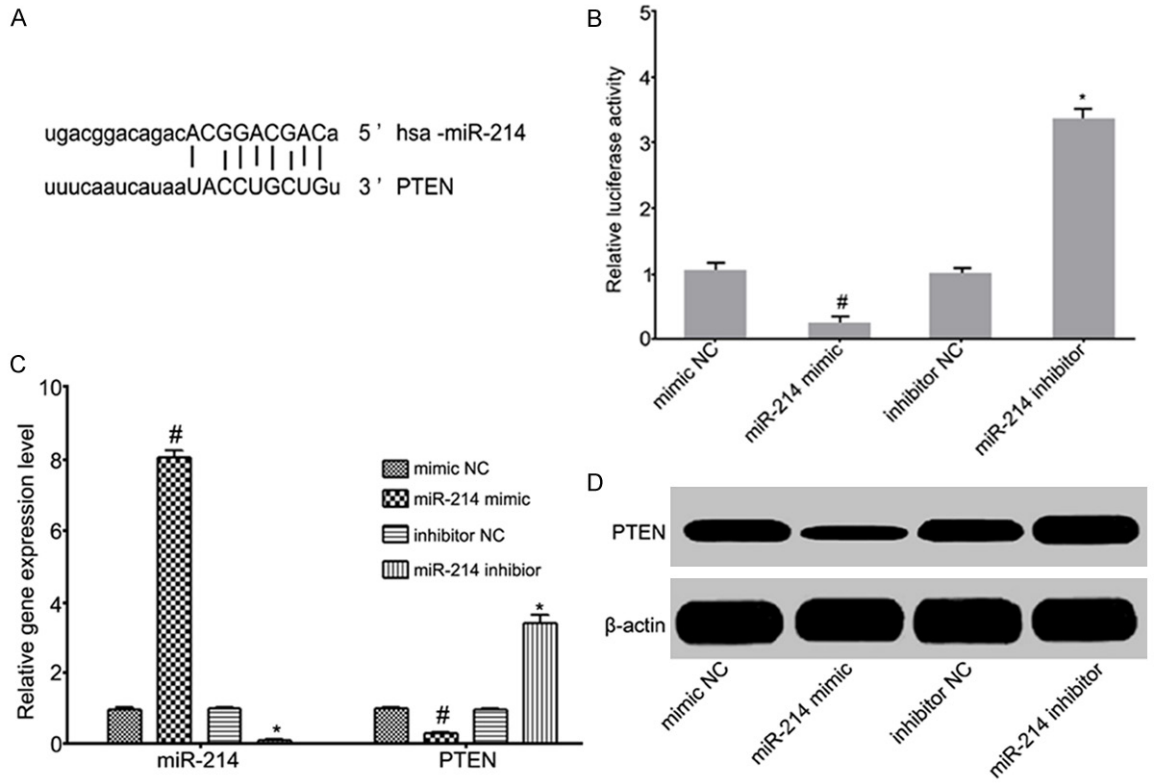

Figure 2. miR-214 targeted regulation on PTEN expression. A: Binding sites between miR-214 and 3'-UTR of PTEN mRNA; B: Dual luciferase reporter gene assay; C: qRT-PCR for miR-214 and PTEN expression; D: Western blot for protein expression. #, P<0.05 compared to mimic NC; *, P<0.05 compared to inhibitor NC.

pIRES2-Scramble, pIRES2-PTEN and miR-214 inhibitor+pIRES2-PTEN group. 72 h later cells were collected for assay.

qRT-PCR for gene expression

cDNA was synthesized in a 10 μL system including 1 μg total RNA, 2 μL RT buffer (5×), 0.5 μL oligo dT+ random primer mix, 0.5 μL RT enzyme mix, 0.5 μL RNase inhibitor, and ddH2O. The reaction conditions were: 37°C for 15 min, followed by 98°C 5 min. cDNA products were kept at -20°C fridge. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase using primers (miR-214P_F: 5'-GGACA GGACG CACAG TCA-3'; miR-214P_R: 5'-CAGAC GAGGC TCCGT GGT-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; PTENP_F: 5'-CTGGT CTGCC AGCTA AAGGT-3'; PTENP_R: 5'-TCACC ACACA CAGGT AACGG-3'; β-actin P_F: 5'-GAACC CTAAG GCCAA C-3'; β-actin P_R: 5'-TGTCA CGCAC GATTT CC-3'; In a PCR system with 10 μL total volume, we added 4.5 μL 2× SYBR Green Mixture, 1.0 μL of forward/reverse primer (at 2.5 μm/L), 1 μL cDNA, and 3.0 μL ddH2O. PCR conditions were: 95°C for 15 s, 60°C for 30 s and 74°C for 30 s. The reaction was performed on Bio-Rad CFX96 fluorescent quantitative PCR cycler for 40 cycles to collect fluorescent data.

Western blot

Proteins were extracted and quantified. 60 μg protein samples were separated by 10% SDS-PAGE for 3 h, and were transferred to PVDF membrane for 1.5 h. The membrane was blocked in 5% defatted milk powder for 60 min, followed by primary antibody (anti-PTEN at 1:100, anti-p-AKT at 1:100 or anti-β-actin at 1:800) incubation at 4°C overnight. By PBST washing (5 min X 3 times), HRP-labelled secondary antibody (1:10,000 dilution) was added for 60 min incubation. After PBST rinsing for three times (5 min each), ECL reagent was added for 2~3 min dark incubation. The membrane was then exposure in dark and scanned for data analysis.

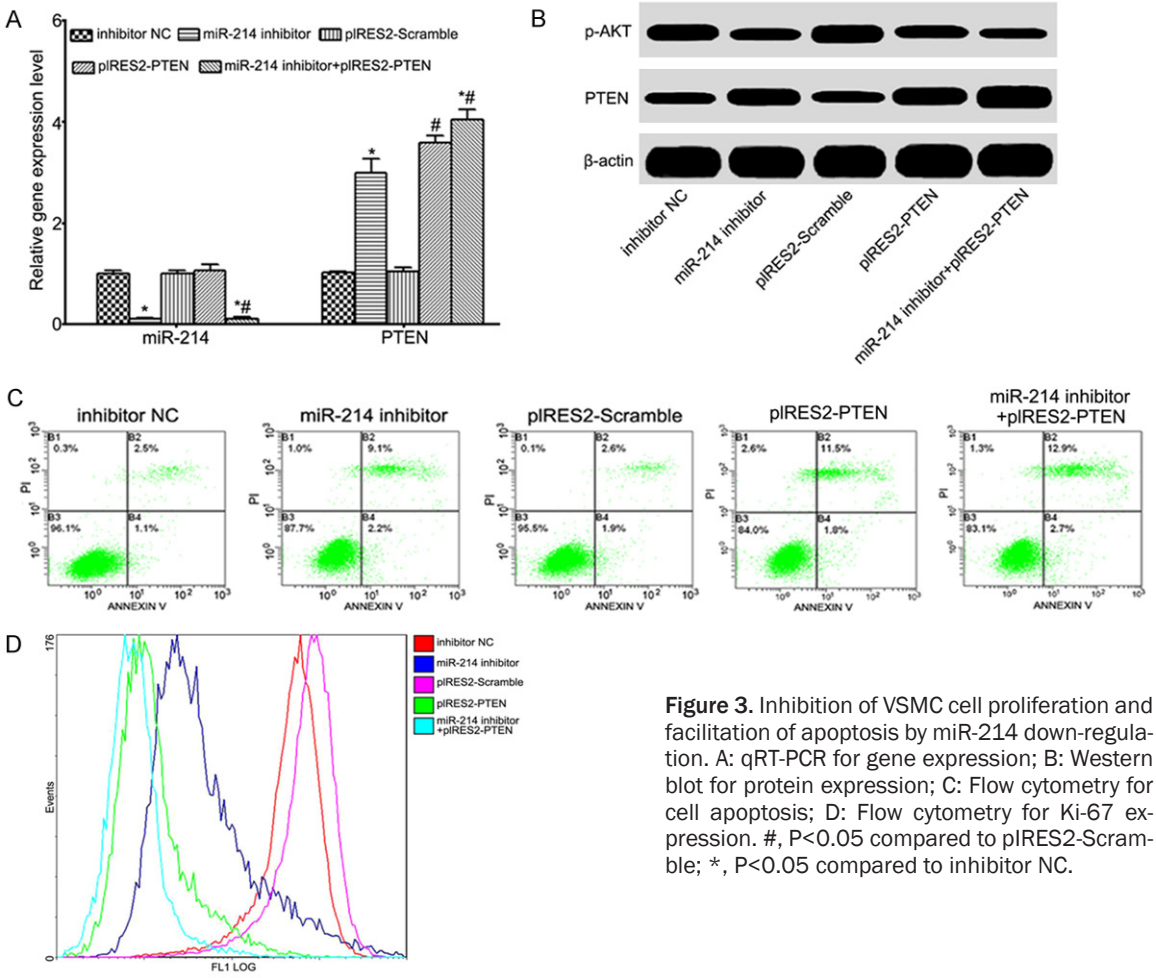

Figure 3. Inhibition of VSMC cell proliferation and facilitation of apoptosis by miR-214 down-regulation. A: qRT-PCR for gene expression; B: Western blot for protein expression; C: Flow cytometry for cell apoptosis; D: Flow cytometry for Ki-67 expression. #, P<0.05 compared to pIRES2-Scramble; *, P<0.05 compared to inhibitor NC.

Flow cytometry for cell apoptosis

Cells were collected by centrifugation, and were then washed in PBS twice. 100 μL Binding Buffer was used to re-suspend cells. The mixture was added with 5 μL Annexin V-FITC and 5 μL PI staining solution. After gentle mixture, the mixture was incubated in dark for 10 min, with the addition of 400 μL 1× Binidng Buffer, and was immediately loaded for online testing in Beckman FC500MCL flow cytometry apparatus.

Flow cytometry for Ki-67 expression

VSMC cells were collected from all groups, and were rinsed twice in PBS containing 2% FBS. After fixation in 4% paraformaldehyde for 20 min, cells were treated using PBS containing 0.1% Triton X-100. PE labelled Ki-67 antibody was added for 4°C dark incubation for 40 min, followed by twice rinsing in PBS containing 2% FBS. Cells were loaded for online testing in Beckman FC500MCL flow cytometry apparatus.

Statistical analysis

SPSS18.0 software was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Student t-test was used to compare measurement data between groups. A statistical significance was defined when P<0.05.

Results

SHR rats had elevated miR-214 expression and lower PTEN expression in vascular middle membrane tissues

qRT-PCR results showed significantly elevated miR-214 expression in SHR rat vascular middle membrane tissues compared to WKY rats with

Table 1. Blood pressure of SHR rats before and after treatment

Treatment	SBP (mmHg)		DBP (mmHg)	
	Before	After	Before	After
Antagomir-214	195.5±16.7	141.8±13.7*	101.9±9.3	89.5±7.6*
Antagomir-control	196.3±18.2	195.7±17.2	100.8±8.9	99.4±8.1

Note: *, P<0.05 compared to those before injection.

normal blood pressure, whilst PTEN mRNA expression was significantly decreased (**Figure 1A**). Western blot results showed significantly decreased PTEN protein expression in SHR rat vascular middle membrane tissues compared to WKY rats, plus remarkably enhanced phosphorylation activity of AKT proteins (**Figure 1B**). These results indicated the possible involvement of miR-214 and PTEN dysregulation in occurrence of hypertension.

miR-214 targeted regulation on PTEN expression

The online prediction results of microRNA.org indicated complementary binding sites between miR-214 and 3'-UTR of PTEN mRNA (**Figure 2A**). Dual luciferase reporter gene assay revealed that the transfection of miR-214 mimic or miR-214 inhibitor significantly decreased or increased relative luciferase activity inside HEK293 cells (**Figure 2B**), and depressed or increased PTEN expression in VSMC cells respectively (**Figure 2C** and **2D**). These results demonstrated that miR-214 could targeted 3'-UTR of PTEN mRNA and regulated its gene expression.

Decrease of miR-214 expression facilitated in vitro VSMC cell apoptosis and inhibited their proliferation

Transfection of miR-214 inhibitor and/or PTEN over-expression plasmid significantly elevated PTEN mRNA and protein in VSMC cells (**Figure 3A** and **3B**), and potentiated phosphorylation activity of AKT (**Figure 3B**), increased cell apoptosis (**Figure 3C**), and weakened proliferation activity (**Figure 3D**).

Down-regulation of miR-214 decreased blood pressure of SHR rats

In vivo injection of micr*OFF*™ antagnomir-214 significantly decreased both SBP and DBP of SHR rats. Injection of micr*OFF*™ antagnomir-control did not obtain significant effects (**Table 1**). Further assay showed that injection of micr*OFF*™ antagnomir-214 significantly depressed PTEN mRNA (**Figure 4A**) and protein (**Figure 4B**) levels in vascular middle membrane of SHR rats, indicating that antagomir-214 could antagonize hypertension via depressing PTEN expression.

Discussion

Due to improvement of life styles, transition of diet habit, fastened life rhythm, higher working pressure, irregular circadian cycle, and aging population, the incidence of hypertension is growing by years [14]. It is estimated that about 153 million people suffered from hypertension, among those about 2.33 million died from hypertension-induced cardiovascular complications [15]. Major pathology basis of hypertension is the persistent elevation of peripheral vascular resistance and tension caused by abnormal activity of vascular constriction/dilation, with VSMC cells playing an important role in maintaining normal constriction/dilation of vessels [16]. VSMC exists under vascular inner membrane endothelial cells, and is the sole cell component inside vascular middle membrane layer. With multiple functions including constriction, synthesis and secretion of proteins, it can maintain vascular wall tension via persistent constriction [17]. Under pathology conditions, or the induction by certain cytokines or growth factors, VSMC presents phenotype transition from constriction phase to synthesis phase, thus acquiring the ability of abnormal proliferation ability with the loss of constriction ability, and migrating from vascular middle membrane to cleft under inner membrane, and releasing abundant cytokines, extracellular matrix and vascular activity substances via autocrine and paracrine manner, thus consisting common patho-physiology basis of various vascular diseases including hypertension [18], atherosclerosis [19] and re-stenosis after angioplasty. Therefore, the inhibition of over-proliferation of VSMC is probably one important treatment method for hypertension.

PTEN locates on chromosome 10q23.3, and transcribes into mRNA with 515 kb as one member of protein tyrosine phosphatase (PTP)

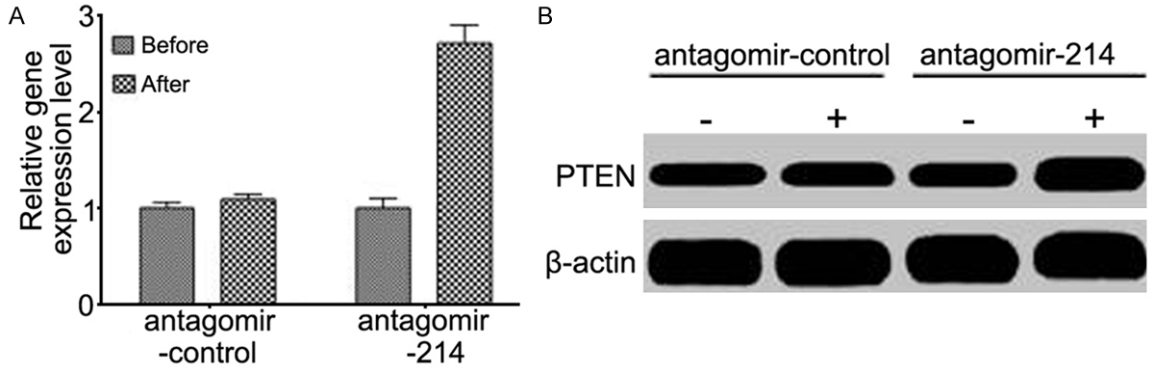

Figure 4. Down-regulation of miR-214 lowered SHR rat blood pressure. A: qRT-PCR for PTEN mRNA expression; B: Western blot for protein expression. *, P<0.05 compared to those before injection. -, before injection; +, after injection.

gene family [20]. Under certain stimuli, phosphoinositide 3-kinase (PI3K) can catalyze the transformation of phosphatidylinositol 4,5-triphosphate (PIP2) to produce phosphatidylinositol 3, 4, 5-triphospate (PIP3), which directly phosphorylates and activates AKT for further activating downstream signal molecules, plus the up-regulation of cell survival factor, cell cycle trophic factor, and down-regulation of anti-apoptotic factors, thus participating in facilitation of cell proliferation and anti-apoptotic effects [21]. PIP3 is the most important substrate of PTEN, which can inhibit phosphorylation of AKT signal molecule and activation of downstream signal pathway via suppressing PI3K through PIP3 phosphorylation [22]. Moreover, PTEN can also directly affects AKT to remove phosphate group on serine/tyrosine residue and inhibit phosphatase activity, thus suppressing PI3K/AKT signal pathway activity, and regulating cell proliferation, cell cycle and apoptosis [23]. Previous study found that PTEN can inhibit PI3K/AKT signal pathway activity and prevent cells to go beyond G1-S phase checkpoint, and can also up-regulate p27Kip1 expression via regulating PI3K/AKT signal pathway, thus arresting cell cycle at G0/G1 phase [24, 25]. PTEN can down-regulate PI3K/AKT signal pathway to down-regulate expression of anti-apoptotic gene BCL-2, thus facilitating cell apoptosis [26]. PTEN also plays a crucial role in negative regulation of PI3K/AKT signal pathway and decrease NF-κB transcription activity for inhibiting cell proliferation [27]. Various studies showed the correlation between pTEN expression or function deficits and pathogenesis of pulmonary artery hypertension, but leaving its role in hypertension occurrence largely unclear [7, 8]. Abnormal elevation of miR-214 expression was also demonstrated to be correlated with occurrence of pulmonary artery hypertension, but leaving its role in hypertension pathogenesis unclear [12, 13]. Bioinformatics analysis revealed complementary binding pairs between miR-214 and 3'-UTR or PTEN mRNA. This study thus investigated if miR-214 played a role in regulating PTEN expression, affecting VSMC proliferation, apoptosis and pathogenesis of hypertension.

Test results showed significantly elevated miR-214 expression in vascular middle membrane tissues of SHR rats compared to WKY rats with normal blood pressure, plus lower PTEN expression and enhanced phosphorylation level of AKT proteins, indicating that abnormal expression of miR-214 and PTEN plus AKT phosphorylation activity might participate in occurrence of hypertension. Ravi et al used monocrotaline (MCT) and hypoxia to induce rat pulmonary artery hypertensive model [8]. Results showed significantly lowered PTEN expression in VSMC of model rats, plus higher p-AKT expression, lower expression of cell cycle modulatory gene p53 and p27, and elevated cyclin-D1 expression, indicating the possible relationship between lower PTEN expression in VSMC and hypertension. Horita et al found significantly lower PTEN expression in VSMC cells of hypoxia-induced pulmonary artery hypertensive rats [7]. The knockout of PTEN significantly facilitated occurrence and progression of pulmonary artery hypertension, plus the correlation between lower PTEN expression and higher proliferation of VSMC, indicating the role of

PTEN in inhibiting VSMC proliferation and impeding hypertension pathogenesis. In a by-pass hypertensive model, it is observed remarkably lower PTEN expression in vascular middle membrane in SHR rats compared to WKY rats with normal blood pressure, sharing common etiology mechanism with Ravi et al [8] and Horita et al [7]. Sahoo et al found elevated miR-214 expression in VSMC cells in pulmonary artery hypertensive patients compared to healthy controlled populations [28]. Liu et al also found higher miR-214 expression in pulmonary artery VSMC cells in pulmonary artery hypertensive patients [12]. This study observed significantly elevated miR-214 expression in vascular middle membrane of SHR rats compared to that of WKY rats with normal blood pressure, possibly sharing common mechanism with Sahoo et al [28] and Liu et al [12] who found the participation of miR-214 up-regulation in hypertension. Dual luciferase reporter gene assay showed that transfection of miR-214 mimic or miR-214 inhibitor significantly decreased or elevated relative luciferase activity of HEK293 cells, and decreased or increased PTEN expression of VSMC, confirming the targeted regulation of miR-214 on PTEN expression. The transfection of miR-214 inhibitor and/or PTEN over-expression plasmid all significantly elevated PTEN expression level in VSMC. Meanwhile phosphorylation of AKT protein was significantly lowered, accompanied with elevated cell apoptosis plus inhibited proliferation activity. Liu et al found that transfection of miR-214 mimic targeted and inhibited CCNL2 expression, further accelerating proliferation of pulmonary artery VSMC cells in pulmonary artery hypertensive patients [12]. The transfection of miR-214 inhibitor significantly inhibited its proliferation. Sahoo et al revealed the role of miR-214 in targeted regulation on MEF2C-MYOCD-LMOD1 signal pathway and facilitating proliferation of pulmonary artery VSMC [30], as consistent with our observation showing the role of miR-214 up-regulation in facilitating VSMC proliferation. *In vivo* injection of miR-214 antagonist antagomir-214 lowered DBP and SBP of SHR rats, and elevated PTEN expression in vascular middle membrane. Results showed that antagomir-214 could exert anti-hypertension effects via decreasing PTEN expression.

Conclusion

MiR-214 could enhance PI3K/AKT signal pathway activity and facilitated proliferation of VSMC plus decreasing apoptosis via targeted inhibition on PTEN expression. Antagomir-214 can exert anti-hypertensive effects via decreasing PTEN expression.

Acknowledgements

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Disclosure of conflict of interest

None.

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