Deregulation of microRNA-31a-5p is involved in the development of primary hypertension by suppressing apoptosis of pulmonary artery smooth muscle cells via targeting TP53

QIANG FENG1, TAO TIAN2, JUNFENG LIU3, LI ZHANG4, JIANGANG QI5 and XIAOJUAN LIN6

1Department of Laboratory, The People's Hospital of Tongchuan, Tongchuan, Shaanxi 727000; 2Department of Laboratory, Second Affiliated Hospital of Shaanxi Chinese Traditional Medicine, Xianyang, Shaanxi 712000; Departments of 3Infection and 4Gynecology and Obstetrics, The People's Hospital of Tongchuan; 5Department of Laboratory, Tongchuan Hospital of Chinese Traditional Medicine; 6Department of Cardiology, The People's Hospital of Tongchuan, Tongchuan, Shaanxi 727000, P.R. China

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Correspondence to: Dr Xiaojuan Lin, Department of Cardiology, The People's Hospital of Tongchuan, 12 Jiankang Road, Tongchuan, Shaanxi 727000, P.R. China
E-mail: rno31ap53@163.com

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Abstract. The present study aimed to identify the association between microRNA (miRNA/miR)-31a-5p and the development of hypertension, and its potential molecular mechanism. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were performed to validate the candidate miRNA and genes involved in hypertension, following which an online miRNA database search, luciferase assay, and RT-qPCR and western blot analyses were performed to confirm the interaction between miR-31a-5p and TP53. A MTT assay and flow cytometric analysis were utilized to determine the effect of miR-31a-5p on cell growth and apoptosis. The results revealed that miR-31a-5p and TP53 were the candidate miRNA and gene regulating hypertension, and that TP53 was the virtual target gene of miR-31a-5p with a binding site located in the TP53 3' untranslated region (3'UTR). It was confirmed by luciferase activity that miR-31a-5p markedly reduced the luciferase activity of the Luc-wild-type-TP53-3'UTR, whereas the mutated putative miR-31a-5p binding located on the TP53-3'UTR was found to eliminate such an inhibitory effect. miR-31a-5p had no effect on specificity protein 1, E2F transcription factor 2 or forkhead box P3 luciferase activity. Smooth muscle cells collected from spontaneously hypertensive rats treated with gold nano-particles containing anti-rno-miR-31a-5p exhibited a lower growth rate and a higher apoptotic rate. The results of the RT-qPCR and western blot analyses showed that miR-31a-5p negatively regulated the expression of TP53, and transfection with the hsa-miR-31a-5p mimic significantly promoted cell growth and inhibited cell apoptosis, whereas transfection with the anti-hsa-miR-31a-5p mimic significantly suppressed cell growth and induced cell apoptosis. Taken together, these findings indicated that miR-31a-5p is involved in hypertension via the accelerated proliferation of arterial smooth muscle cells and inhibition of apoptosis through targeting TP53.

Introduction

High blood pressure (HBP) is a risk factor for cardiovascular disorders, including chronic kidney disease, congestive heart failure, myocardial infarction and stroke, and is a leading contributor to rates of mortality and morbidity in the world. Substantial progress has been made in the treatment of HBP via the application of α-adrenoreceptor antagonists, β-blockers, calcium channel blockers, renin-angiotensin system inhibitors and diuretics. Despite the availability of a variety of blood pressure drugs, a larger number of patients with HBP are unable to get their blood pressure under control. It was estimated that only 51.9% of patients with HBP in the United States had their blood pressure under control (<140/90 mm Hg) between 2011 and 2012 (1).

As a basic composition of vascular walls, vascular smooth muscle cells (VSMCs) have a well-differentiated contractile phenotype, which is important to maintain vascular tone (2). The increased proliferation of VSMCs is associated with HBP (3,4). Vascular inflammation is considered to be involved in vascular remodeling in a variety of cardiovascular disorders, including atherosclerosis and HBP (5).

The tumor inhibitor protein p53 (protein product of TP53, also known as p53) is known to be involved in the development of restenosis and atherosclerosis, VSMC growth and cell death (6). A previous study reported that elevated nuclear translocation of p53 in cancer cells was observed in response to therapy with microtubule stabilizing agents (MTSAs) (7). It is widely known that the microtubule stabilization triggered by MTSAs is the primary mechanism attributable to the elevation in the association of p53 with microtubules and its nuclear export, which is associated with the apoptotic pathway dependent...
on p53 (7). Due to the tumor inhibitory functions of p53, this protein has attracted attention from the pulmonary arterial hypertension community (8). Mizuno et al revealed that mice with p53 knockout formed more serious pulmonary hypertension in response to chronic hypoxia than wild-type mice (9).

As small and non-coding RNA molecules, microRNAs (miRNAs) consist of ~22 nucleotides and binding to their target mRNAs to suppress translation, which have a key regulatory role in eukaryotic genes, particularly in cell proliferation, differentiation and apoptosis (10). Often, miRNAs can bind to the 3′ untranslated region (3′UTR) of the mRNAs of target genes in an imperfect or perfect complementary manner, leading to translational repression or mRNA degradation (11). Increasing data have revealed that dysregulated miRNAs are associated with cardiovascular disorders, including vascular atherosclerosis, heart failure and cardiac hypertrophy (12). Baseline gene expression levels of miRNA-26b, miRNA-499, miRNA-208b, miRNA-21, miRNA-133a and miRNA-1 have been determined in peripheral blood mononuclear cells (PBMCs), cells identified to be important in the pathophysiology of target organ injury (13). These miRNAs were selected as they have a different expression profile in HBP, and have been associated with heart and vascular remodeling (14). The expression of miRNAs in the PBMCs of patients has been investigated, as PBMCs are of important in the cardiovascular complications of HBP (15).

A previous study demonstrated the differential expression of miR-31a-5p in the smooth muscle cells collected from an animal model of primary hypertension, compared with the control, and it has been reported that dysregulated p53 is associated with the molecular mechanism of smooth muscle cell apoptosis (16-18). The present study performed a search on an online miRNA database and found that miR-31a-5p virtually targets p53. In the present study, miR-31a-5p was found to target p53, and the association of p53 and miR-31a-5p in the occurrence of primary hypertension was confirmed.

Materials and methods

Animals. All experiments were performed in 28 male adult spontaneously hypertensive rat (SHR; 16 rats) and normotensive Wistar-Kyoto (WKY; 12 rats) rats (15-16 weeks old, weighing 430±40 g) following the institutional guidelines that comply with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, 2011). All procedures of experiments were approved by the Experimental Animal Care and Use Committee of China Medical University (Shenyang, China). All rats were housed at room temperature (23±2°C) with a 12 h-12 h light/dark cycle, and were provided with a rodent chow diet and drinking water throughout the experiment.

Isolation and culture of pulmonary artery smooth muscle cells (PASMCs). The PASMCs were isolated from tissue samples derived from the rats; forceps were utilized to mince the tissue samples, and 4 mg/ml dispase (Sigma-Aldrich; EMD Millipore, Bedford, MA, USA) was used to digest the tissues for 30 min at 37°C, and subjected to additional incubation for another 5 h. A 40 µm cell strainer (BD Falcon, Bedford, MA, USA) was utilized to filter the dissociated cell suspension. Centrifugation was performed for 15 min at 107.3 x g at 4°C to yield the cells. DMEM with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 µg/ml streptomycin and 100 U/ml penicillin was utilized to incubate the cells under a humidified atmosphere with 5% CO2/95% air at 37°C. The medium was replaced at 2-day intervals until the cells were cloned. The cells at passage three were used in subsequent experiments.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized to isolate total RNA from the tissue samples, and the mirVana™ PARIS™ kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was utilized to extract total RNA from the PASMCs, based on the manufacturer's protocol. Subsequently, 8% denaturing polyacrylamide gels were utilized to monitor RNA integrity. TaqMan miRNA assays was performed to perform RT-qPCR analysis, and a high-capacity cDNA archive kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was utilized to reverse transcribe miRNA to cDNA according to the manufacturer's protocol. A NanoDrop instrument (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) was utilized to determine the concentrations of RNA, and U6 served as a control to normalize the expression of miR-31a-5p. SYBR-Green-based detection systems (Applied Biosystems; Thermo Fisher Scientific, Inc.) were utilized to perform the PCR amplifications on a GeneAmp PCR 9700 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol, using Standard Taq Reaction Buffer (10X, 5 µl), dNTPs (10 mM, 1 µl), Forward Primer (10 µM, 1 µl), Reverse Primer (10 µM, 1 µl), Template DNA (10 ng), Taq DNA Polymerase (0.25 µl) and nuclease-free water (50 µl). The primer sequences used were as follows: RT primer, 5′GTGATATCCTAGTGTTGCTGGATCCGGAATTTGACCTGAGTACGACAGCTA-3′; forward, 5′-GGGAGGCAGATGTGGCAG-3′ and reverse, 5′-CAGTGGCTGTCGGT-3′. The thermocycling conditions were as follows: 95°C for 30 sec, 55°C for 30-60 sec, 72°C for 30-60 sec for 30 cycles, and 72°C for 5 min. Melting curve analysis was utilized to confirm the lack of primer dimers and specificity of amplification. The 2^−ΔΔCt method (19) was utilized to analyze the expression of TP53 mRNA and miR-31a-5p. All experiments were run three times.

Cell culture and transfection. Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS, 100 mg/ml streptomycin and 100 U/ml penicillin was utilized to culture the PASMCs under a humidified atmosphere of 5% CO2 at 37°C. Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) was utilized to perform transient transfections of miR-31a-5p mimic, miRNA control, and TP53 small interfering (si)RNA. The sequences were as follows: miR-31a-5p mimic, 5′-AGGCAGAGUGGCUUAGUGCUU-3′; miRNA control, 5′-CAGCUAGUCGAGCU-3′; and reverse, 5′-TTTGGGACTTGAAGCATCTG-3′. All experiments were performed in triplicate.
Cell proliferation assay. The PASMCs were seeded into 24-well plates at a final concentration of 2x10^3 cells per well. An MTT assay was performed to evaluate cell viability 24, 48 and 72 h post-MTT addition. An ELISA reader (ELX-800 type; Bio-Tek Instruments, Inc., Winooski, VT, USA) was used to measure the optical density of each well at 570 nm to quantify cell proliferation. Each experiment was repeated three times.

Luciferase assay. RT-PCR was performed to amplify the p53 3’UTR containing the putative or mutated binding site of miR-31a-5p. The thermocycling conditions were as follows: 25°C for 10 min, 42°C for 50 min, 70°C for 15 min and 37°C for 20 min. The PCR products were then inserted into the hR-luc luciferase coding sequence downstream, which was located in the pmir-RB-REPORT™ vector (Guangzhou RiboBio Co., Ltd.; Guangzhou, China) following standard protocol. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was utilized to co-transfect the cells with the luciferase constructs and miR-31a-5p mimic or negative control. At 48 h post-transfection, a Dual Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA) was utilized to measure the luciferase activity of Renilla and Firefly based on the manufacturer’s protocol. The Renilla luciferase activity was normalized to Firefly luciferase activity. Three independent experiments were performed.

Western blot analysis. RIPA buffer (Sigma-Aldrich; EMD Millipore) was utilized to extract protein from the cells at 48 h post-transfection following the standard protocol. The lysates were centrifuged at 13,000 g for 15 min at 4°C to collect the upper supernatant. The bicinchoninic acid method was utilized to measure the protein concentration. SDS-polyacrylamide gels (12.5%; Invitrogen; Thermo Fisher Scientific, Inc.) were utilized to electrophorese 30 µg of the extracted protein, which were then blotted onto polyvinylidene difluoride membranes (EMD Millipore, Bedford), followed by blocking with 5% non-fat milk. Specific primary antibodies against p53 (cat. no. 9282T; 1:5,000; Cell Signaling Technology, Inc., Beverly, MA, USA) and against β-actin (cat. no. 4967S; 1:80,000, Sigma; EMD Millipore) were added for incubation with the membrane for 12 h at 4°C, and TBST buffer was utilized to wash the membrane three times. Two independent antibody conjugates against p53, specificity protein 1 (SP1), E2F transcription factor 2 (E2F2) and forkhead box P3 (FOXP3), which were identified by searching the online miRNA database, (www.mirdb.org). The expression of these candidate target genes were determined and compared between cells collected from the SHR and WKY groups.

Analysis of apoptosis. At 48 h post-transfection, the PASMCs were harvested, and PBS was utilized to wash cells. A FITC-Annexin V/Propidium Iodide Apoptosis Detection kit (BestBio, Shanghai, China) was used to resuspend and stain the cells according to the manufacturer’s protocol. Flow cytometry (BD FACScanto II; BD Biosciences, San Jose, USA) was used to analyze cell apoptosis. Each experiment was performed three times.

Results

Identifying candidate miRNAs involved in hypertension. The SHR model is a genetic animal model for essential hypertension, which shows elevated blood pressure compared with normotensive WKY rats. RT-qPCR analysis was used to compare the expression of the candidate miRNAs (rno-miR-31a-5p, rno-miR-31a-3p and rno-miR-146a-5p) between the SHR and WKY rats, as shown in Fig. 1. Only rno-miR-31a-5p showed differential expression in the SHR rats in comparison with that in WKY rats, whereas no significant difference in levels of rno-miR-31a-3p and rno-miR-146a-5p were observed between the SHR and WKY rats.

Identifying candidate target genes of rno-miR-31a-5p. The PASMCs were collected from the SHR and WKY rats, and RT-qPCR analysis and western blot analysis were performed to examine candidate target genes, including p53, specificity protein 1 (SP1), E2F transcription factor 2 (E2F2) and forkhead box P3 (FOX3P3). These candidate target genes were identified by searching the online miRNA database, (www.mirdb.org). The expression of these candidate target genes were determined and compared between cells collected from the SHR and WKY groups. As shown in Fig. 2, only the mRNA and protein (Fig. 2A) levels of p53 were decreased in the SHR group, compared with those in the WKY group, whereas the mRNA and protein levels of SP1 (Fig. 2B), E2F2 (Fig. 2C) and FOX3P (Fig. 2D) were comparable between the SHR and WKY groups.

p53 is a candidate target gene of miR-31a-5p. To further validate p53 as a direct target of rno-miR-31a-5p, vectors
Alteration of the expression of rno-miR-31a-5p affects the expression of p53, and proliferation and apoptosis of PASMCs human arterial smooth muscle cells. To further examine the role of miR-31-5p in the control of cell proliferation and apoptosis in PASMCs, the cells were transfected with the hsa-miR-31a-5p mimic, anti-hsa-miR-31a-5p mimic or the scramble controls. RT-qPCR analysis, western blot analysis, an MTT assay and flow cytometry were performed to determine the levels of miR-31-5p and p53, and the proliferation and apoptosis of the differently treated cells. As shown in Fig. 5, the anti-hsa-miR-31a-5p mimic (Fig. 5A) reduced the level of miR-31a-5p. The mRNA and protein levels of p53 in the cells were upregulated subsequent to transfect with the anti-hsa-miR-31a-5p mimic (Fig. 5B). The downregulation of miR-31a-5p by transfection with the anti-hsa-miR-31a-5p mimic inhibited the viability of arterial smooth muscle cells (Fig. 5C) and promoted the apoptosis of arterial smooth muscle cells (Fig. 5D and E), but did not affect the cell cycle status (Fig. 5F). The hsa-miR-31a-5p mimic (Fig. 6A) increased the level of miR-31a-5p. The mRNA and protein levels of p53 in the cells were downregulated following transfection with the hsa-miR-31a-5p mimic (Fig. 6B). The overexpression of miR-31a-5p significantly promoted the growth of the arterial smooth muscle cells (Fig. 6C) and significantly inhibited the apoptosis of the cells (Fig. 6D and E), but did not affect the cell cycle status (Fig. 6F).

Collectively, these findings indicated that miR-31a-5p accelerated the proliferation of arterial smooth muscle cells and inhibited apoptosis via targeting p53.

Discussion

It has been shown that miRNAs, which modulate the proliferation or migration of endothelial progenitor cells and embryonic/mesenchymal stem cells (miR-702, miR-221/222 and miR-31), and reduce stem cell apoptosis and cancer (miR-31 and miR-702), are increased in hypertrophic RV, compared with those in controls according to unbiased quantitative miR microarray analysis (20,21). It was previously revealed that only three miRs (miR-31a-5p, miR-31a-3p and miR-208b) were increased in the LV of PAH rats (22). The levels of C-kit and miR-31 were elevated in hypertrophic RV of PAH rats, compared with those in control rats, and miR-31 was elevated in the RV of PAH rats, which acts as a compensatory mechanism to decrease the reduction in capillary density, which is associated with the failing hearts (23). In the present study, candidate miRNAs (rno-miR-31a-5p, rno-miR-31a-3p and rno-miR-146a-5p) were investigated by comparing the expression levels between PASMCs from SHR and WKY rats, and it was revealed that only rno-miR-31a-5p was significantly upregulated in the SHR rats, compared with the WKY rats. Previously, studies have shown that miR-31 is involved in VSMC proliferation, angiogenesis and...
tumor metastasis, although miR-31 was found to be the most increased miRNA following acute myocardial infarction in rats (24-26). miR-31-induced cardioprotection was eliminated when the activation of nuclear factor (NF)-κB was suppressed by Adv-dnIκBα during the ischemia/reperfusion (I/R) process, revealing that diverse mechanisms may be involved in the miR-31/PKCε signaling-induced and I/R-induced activation of NF-κB in cardiac myocytes (27).

Patients with primary hypertension, a hereditary polygenic disease, eventually develop complications, including nephrosclerosis, cardiovascular remodeling and stroke. These complications are practical targets for the treatment of underlying HBP with blood pressure drugs. SHR rats, a genetic animal model for underlying hypertension, indicates poorer growth of cardiovascular organs compared with normotensive WKY rats (28). SHR-derived VSMCs in culture exhibit accelerated entry into the S phase of the cell cycle, aberrant contact suppression, a higher specific growth rate, and nonspecific hyperproliferation responding to a variety of growth factors, compared with cells from WKY rats (29). In the present study, candidate genes of miR-31a-5p were investigated using an online miRNA database, and four genes were found, including p53, SP1, E2F2 and FOXP3, which may be target genes of miR-31a-5p with a complementary binding site of miR-31a-5p located in their 3'UTR respectively. It was then found that only the mRNA and protein levels of p53 were differential in the SHR group, compared with those in the WKY group, whereas the mRNA and protein levels of SP1, E2F2 and FOXP3 in the SHR group did not differ significantly compared with those in the WKY group, and this regulatory association was further confirmed by the results of the luciferase assay.

\[ \text{p53, a tumor inhibitor, is a key transcription factor that modulates several cellular processes. It can suppress cell proliferation by triggering cell cycle arrest in the G1, G2 and S} \]
phases of the cell cycle (30); its expression is associated with elevated cell apoptosis in vitro and in vivo (31). p53 predominantly acts as a transcription factor, which can induce various anti-proliferative programs by the inhibition or activation of critical genes or effects (32). For VSMCs, the downregulation of p53 occurs prior to VSMC migration and proliferation (33). A hypermethylation status has been observed in p53 promoter region when treatment by Hcy, which indicates a causative function for VSMC proliferation (17). Therefore, p53 is considered a potent negative modulator of cell proliferation, including that of VSMCs. The p53 tumor inhibitor protein is stimulated in response to various cellular stresses, including nucleotide depletion, oncogene activation and DNA damage, with p21 and MDM2 being the most well-known examples of these targets (34). Indirect p53-mediated suppression is also involved via stimulation of its direct transcriptional target, known as p21 (35). Representing the Ink4a/Cip1 family of cyclin-dependent kinase (CDK) inhibitors, p21 suppressors bind to and suppress CDK4 and CDK6/cyclin D complexes to trigger cell-cycle arrest, leading to the activation and de-phosphorylation of retinoblastoma pocket proteins, which act together with E2F transcription factors to inhibit genes associated with the cell cycle, including hTERT, EZH2 and cHK1, which are p53-inhibition targets modulated by p21 (36,37).

Figure 4. Arterial smooth muscle cells from spontaneously hypertensive rats treated with gold nanoparticles containing scramble control or anti-rno-miR-31a-5p mimic were collected. (A) Anti-rno-miR-31a-5p inhibited the expression of miR-31a-5p. (B) Anti-rno-miR-31a-5p increased the expression of TP53. (C) Anti-rno-miR-31a-5p downregulated cell viability. (D) Anti-rno-miR-31a-5p induced cell apoptosis. (E) Representative flow cytometry plot for apoptosis status. (F) Treatment did not affect the cell cycle status of the cells. miR, microRNA.
apoptosis of the PASMCs. Finally, RT-qPCR analysis, western blot analysis, an MTT assay, and flow cytometry were used to determine the levels of miR-31-5p and TP53, and the proliferation and apoptosis of cells transfected with anti-hsa-miR-31a-5p mimic. (A) Anti-rno-miR-31a-5p inhibited the expression of miR-31a-5p. (B) Anti-rno-miR-31a-5p increased the expression of TP53. (C) Anti-rno-miR-31a-5p downregulated cell viability. (D) Anti-rno-miR-31a-5p induced cell apoptosis. (E) Representative flow cytometry plot for apoptosis status. (F) Treatment did not affect the cell cycle status of the cells. miR, microRNA.

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The effects of miR-31 have been associated with the status of p53. miR-31 acts as an inhibitor only in tumor cells that harbor mutant p53, which indicates miR-31 as a target for therapy in patients with p53-deficient tumors (38). Of note, the p53 mutation is an early indicator in esophageal squamous cell cancer (ESCC); additionally, alterations in the p53 status may contribute to context-dependent effects of several molecules, which include microRNAs, including miR-31 (38,39). It has been shown that the inhibitory role of miR-31 in ESCC relies on a deficiency of p21 in addition to modulation by p53 (40).

In conclusion, the findings of the present study demonstrated that the deregulation of miR-31a-5p was associated with the risk of hypertension by suppressing the apoptosis of arterial smooth muscle cells. It was found that p53, a well-known tumor suppressor, was a direct target gene of miR-31a-5p, which was important in the apoptosis of arterial smooth muscle cells and was involved in the pathogenesis of hypertension. Therefore, miR-31a-5p may be a novel therapeutic strategy for the treatment of hypertension.
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Availability of data and materials

The datasets generated and analyzed in the present study are included in this published article.

Authors’ contributions

QF: Study planning, data collection, data analysis and interpretation, preparation of the manuscript and literature analysis. TT: Study planning, data collection, data analysis and interpretation and literature analysis. JL: Study planning, data collection, data analysis and interpretation and literature analysis. LZ: Data collection, data analysis and interpretation and literature analysis. JQ: Data analysis and interpretation, preparation of the manuscript and literature analysis. XL: Data collection, data analysis and interpretation and funds collection.

Ethics and consent to participate

All procedures of experiments were approved by the Experimental Animal care and Use Committee of China Medical University (Shenyang, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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