Abstract. The aim of the present study was to measure the expression of microRNA (miRNA)-506-3p in the peripheral blood of patients with hypertension and to determine the biological functions and mechanisms of action of miR-506-3p. A total of 61 patients with primary hypertension were included in the present study. Peripheral blood was collected from all patients, as well as 31 healthy subjects who were included in a control group. The expression of miR-506-3p in peripheral blood was determined by reverse transcription-quantitative polymerase chain reaction. Human umbilical vein endothelial cells (HUVECs) were transfected with miR-506-3p mimics or miR-506-3p inhibitor. The proliferation and migration of HUVECs were determined using cell-counting kit 8 and Transwell assays, respectively. The cell cycle and apoptosis of HUVECs were detected by flow cytometry. The expression of Beclin1 (BECN1) protein, a potential target of miR-506-3p, was measured using western blotting. A dual-luciferase reporter assay was performed to determine the interaction between BECN1 and miR-506-3p. It was demonstrated that miR-506-3p expression in the peripheral blood of patients was upregulated and dependent on the severity of hypertension. miR-506-3p overexpression inhibited the proliferation and migration of HUVECs. In addition, miR-506-3p inhibited the transition from the G1 phase to the S-phase in HUVECs. Overexpression of miR-506-3p promoted the apoptosis of HUVECs. Notably, miR-506-3p downregulated the expression of BECN1 by directly binding to its 3'-untranslated region. The present study demonstrated that miR-506-3p expression is elevated in the peripheral blood of patients with hypertension and is associated with the severity of hypertension. By downregulating BECN1 expression, miR-506-3p aggravates injury in vascular endothelial cells by inhibiting the proliferation and migration of HUVECs, as well as promoting their apoptosis.

Introduction

Hypertension is a common chronic cardiovascular disease clinically characterized by an elevated blood pressure and is an independent risk factor for cardiac and cerebral vascular diseases (1-3). The incidence of hypertension increases each year, particularly among young people (4); however, its molecular mechanism of action remains unclear. As an important component of the blood circulation and immune system, vascular endothelial cells are widely involved in biological processes, including blood pressure regulation, blood vessel formation, anticoagulation, fibrinolysis and inflammation (5-7). Previous studies have demonstrated that vascular endothelial cell dysfunction serves an important role in the onset and development of hypertension (8,9). Endothelial cells not only act as a physical barrier but also secrete a variety of substances that affect the function of relaxation and contraction of blood vessels. During sustained hypertension, endothelial cells are damaged and the expression of inflammatory factors is increased (10,11). Therefore, investigating the molecular mechanism of vascular endothelial cells in the onset and development of hypertension may help to improve the diagnosis and treatment of hypertension.

MicroRNA (miRNA) are a class of non-coding small RNA molecules consisting of 18-23 nucleotides. miRNA regulates the expression of target mRNA at the post-transcriptional level by binding to the 3'-untranslated region (UTR) of target gene mRNA (12,13). miRNA participates in a wide variety of biological processes, including cell proliferation, differentiation, apoptosis, energy metabolism, hormone secretion and development (14,15). miRNA serves an important biological role in cardiovascular diseases and affects the progression of hypertension by regulating a variety of tissues and organs, including myocardial tissue, vascular wall tissue, fundus tissue and kidney (16). Furthermore, miRNA serves important regulatory roles in cardiac hypertrophy and heart

Overexpression of microRNA-506-3p aggravates the injury of vascular endothelial cells in patients with hypertension by downregulating Beclin1 expression

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failure (17,18) and its expression is closely associated with arrhythmia and atherosclerotic plaque formation. As miRNA expression is stable in peripheral blood, it may act as a potential diagnostic marker for various diseases, including myocardial infarction and cancer (19). The upregulation of miR-1 levels in the peripheral blood is closely associated with myocardial injury (20). However, the mechanism by which miRNA induces endothelial injury in hypertension remains unclear.

It has been demonstrated that the recently identified miRNA molecule miR-506 is closely associated with proliferation, drug resistance, invasion and metastasis in tumors. The upregulation of miR-506 increases drug resistance in colon cancer cells (21) and in neuroglioma, miR-506 inhibits the proliferation and metastasis of tumor cells by targeting signal transducer and activator of transcription 3 (22). In addition, miR-506 participates in angiogenesis, suggesting that it may serve a role in cardiovascular disease (23). Beclin1 (BECN1) is a key gene in the regulation of autophagy activity and participates in the onset and development of different tumors by directly regulating autophagy activity (24). In most tumors, BECN1 expression is downregulated and BECN1 overexpression inhibits tumor growth (25,26). It remains unclear whether miR-506-3p is associated with BECN1 expression. Therefore, the aim of the present study was to investigate the effect of miR-506-3p in the onset and development of hypertension at the clinical and cellular levels.

Materials and methods

Patients. A total of 61 patients with primary hypertension admitted to Zaozhuang Municipal Hospital (Zaozhuang, China) between December 2014 and January 2016 were included in the present study. Peripheral blood was collected from all patients, as well as 31 healthy subjects who were included in a control group. The 61 patients included 34 males and 27 females, with a mean age of 62.5 years (median, 61 years; range, 46–81 years). Diagnosis of hypertension was determined when systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥100 mmHg without the use of antihypertensive drugs, in accordance with the 2010 Chinese Guidelines for the Management of Hypertension (27). The 61 patients were divided into grades I, II and III according to the severity of hypertension (27). Patients with grade I had mild hypertension (systolic blood pressure of 140–159 mmHg and/or diastolic blood pressure of 90–99 mmHg), patients with grade II had moderate hypertension (systolic blood pressure of 160–179 mmHg and/or diastolic blood pressure of 100–109 mmHg) and patients with grade III had severe hypertension (systolic blood pressure of ≥180 mmHg and/or diastolic blood pressure of ≥110 mmHg). There were 23, 27 and 11 patients with grades I, II and III, respectively. The duration of hypertension for each patient was >5 years. All procedures were approved by the Ethics Committee of Zaozhuang Municipal Hospital (Zaozhuang, China) and written informed consent was obtained from all patients or their families.

Cells. Human umbilical vein endothelial cells (HUVECs; Cell Bank; Chinese Academy of Sciences, Shanghai, China) and HEK293T cells (Cell Bank; Chinese Academy of Sciences) were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO2. Upon reaching 70–90% confluence, cells were seeded into 24-well plates at 1x10⁴ cells/well. HUVECs were divided into three groups: A negative control (NC) group, a miR-506-3p mimics group (transfection with miR-506-3p mimics) and a miR-506-3p inhibitor group (co-transfected with miR-506-3p mimics and inhibitor).

Transfection of cells. Prior to transfection, HUVECs were cultured in serum-free DMEM until they reached 90% confluence. In the first vial, 2.5 µl miR-506-3p mimics (25 pmol/µl; miR-506-3p mimics group; HanBio, Shanghai, China; sequences not supplied), or 2.5 µl miR-506-3p mimics (25 pmol/µl; HanBio; sequences not supplied) and 2.5 µl miR-506-3p inhibitor (25 pmol/µl; miR-506-3p inhibitor group; HanBio; sequences not supplied) were mixed with 50 µl Opti Mem I medium (Thermo Fisher Scientific, Inc.). In the second vial, 1 µl Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) was mixed with 50 µl Opti Mem I medium. Vials were left to stand for 5 min, the two vials were then combined and left to stand at room temperature for a further 20 min. Subsequently, mixtures were added to cells in each respective group. After 6 h, the medium was replaced with fresh DMEM containing 10% fetal bovine serum. Following cultivation for 48 h, cells were collected for further assays.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells (1x10⁵) were trypsinized and mixed with 0.5 ml TRIzol (Thermo Fisher Scientific, Inc.) for lysis. Total RNA was then extracted using the phenol chloroform method (28). The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (nanodrop ND2000, Thermo Fisher Scientific, Inc.). Subsequently, cDNA was obtained by reverse transcription using the Reverse Transcription System (Takara Biotechnology, Co., Ltd., Dalian, China) using 0.5 µg RNA and stored at -20°C. The expression of miR-506-3p was determined using a SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology, Co., Ltd.) and U6 (forward, 5'-CTC GCT TCG GCA GCA CA-3'; reverse, 5'-AACGTTCTACGAAATTTGC-3') was used as an internal reference. The reaction system (20 µl) contained 10 µl qRT-PCR-Mix, 0.5 µl upstream primer (5'-GGCACCTTCTGTAGA-3'), 0.5 µl downstream universal primer (provided by the kit), 2 µl cDNA and 7 µl ddH2O. The reaction protocol was: Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation 95°C for 1 min and annealing at 60°C for 30 sec. The reaction was performed on ABI StepOne Plus Real-Time PCR system (Thermo Fisher Scientific, Inc.). The 2-ΔΔCt method (29) was used to calculate the relative expression of miR-506-3p against U6 and each sample was measured in triplicate.

Cell counting kit-8 (CCK-8) assay. At 24 h after transfection, HUVECs were trypsinized and seeded into 96-well plates at a density of 1x10³/well. At 24, 48 and 72 h, the medium was discarded and the cells were washed with sterile phosphate-buffered saline twice. Subsequently, DMEM and 10% CCK-8 reaction reagent (Beyotime Institute of Biotechnology, Haimen, China) were added. Following
incubation at 37°C for 1 h, the absorbance of each well was measured at 490 nm and cell proliferation curves were plotted.

Flow cytometry. At 48 h after transfection, HUVECs were trypsinized, collected and washed twice with precooled phosphate-buffered saline. Cellular apoptosis was measured by flow cytometry using a FITC Annex V apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ, USA), following the manufacturer's instructions. Early apoptotic cells were identified by single-positive staining with Annexin V, necrotic cells were identified by single-positive staining with propidium iodide and late apoptotic cells were identified by double-positive staining with propidium iodide and Annexin V. To determine the cell cycle, a cell cycle assay kit (BD Biosciences) was employed. Cells were incubated with 200 µl liquid A at room temperature for 10 min, followed by 150 µl liquid B at room temperature for 10 min and finally 120 µl liquid C at room temperature in the dark for 10 min. A flow cytometer was used for detection and the results were analyzed using ModFit v3.2 software (http://www.vsh.com).

Transwell assay. Transwell chambers (8 µm; 24-wells; Corning Inc., Corning, NY, USA) were used to evaluate the migration ability of HUVECs. Cells were collected by trypsin digestion and resuspended to a density of 5x10^5 cells/ml using DMEM containing 0.1% bovine serum albumin (Beyotime Institute of Biotechnology). The cell suspension (200 µl) was added to the upper chamber. In the lower chamber, 500 µl DMEM supplemented with 10% fetal bovine serum was added. Following 24 h cultivation, cells in the upper chamber were wiped using a cotton swab. Cells that moved to the other side of the chamber were fixed with 4% formaldehyde at room temperature for 10 min. Following staining with Giemsa at room temperature for 1 h, the number of cells in five randomly selected fields were counted under a light microscope (magnification, x200) and averaged.

Western blot analysis. HUVECs were trypsinized and collected prior to mixing with 100 µl precooled radioimmuno precipitation assay lysis buffer (Beyotime Institute of Biotechnology) containing 1% phenylmethylsulfonyl fluoride for 15 min lysis at 4°C. Subsequently, cells were centrifuged at 12,000 x g and 4°C for 10 min. The supernatant was used to determine protein concentration using a bicinchoninic acid protein concentration determination kit [RTP7102, Real-Times (Beijing) Biotechnology Co., Ltd., Beijing, China]. Subsequently, samples were mixed with 5X loading buffer prior to denaturation in a boiling water bath for 10 min. Protein samples (20 µg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (300 mA, 1 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, membranes were incubated with rabbit anti-human polyclonal primary antibodies for BECN1 (1:1,000; cat. no. ab62557) and GAPDH (1:1,000; cat. no. ab8245; both Abcam, Cambridge, UK) at 4°C overnight. Following six washes with phosphate-buffered saline and Tween-20 (5 min each), membranes were incubated with polyclonal goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:4,000; cat. no. ab6721; Abcam) for 1 h at room temperature prior to six washes with phosphate-buffered saline and Tween-20 (5 min each). Subsequently, membranes were developed using an enhanced chemiluminescence detection kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Image lab v3.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to acquire and analyze imaging signals. Relative protein content was expressed against GAPDH.

Bioinformatics prediction. Bioinformatics prediction is a powerful tool for the study of the functions of miRNA. To determine the regulatory mechanism of BECN1, miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org), PiTa (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and PICTA (http://picтар.mdc-berlin.de/) were used to predict the miRNA molecules that may regulate BECN1. It was demonstrated that miR-506-3p was able to regulate BECN1. miRNA/miR, microRNA; BECN1, Beclin1.
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Results

Expression of miR-506-3p in the peripheral blood of hypertension patients is upregulated and dependent on hypertension staging. To measure the expression of miR-506-3p in the peripheral blood, RT-qPCR was performed. The data indicated that miR-506-3p expression in patients with hypertension was significantly higher compared with the control group (P<0.05; Fig. 2A). Further analysis indicated that the expression of miR-506-3p in patients with grade III hypertension was significantly higher than in patients with grade I or II hypertension (P<0.05; Fig. 2B), whereas miR-506-3p expression did not differ between patients with grade I and II hypertension (P>0.05; Fig. 2B). These results demonstrate that miR-506-3p expression in the peripheral blood of patients with hypertension is upregulated and that this upregulation is dependent on hypertension staging.

Overexpression of miR-506-3p inhibits the proliferation of HUVECs. To determine the effect of miR-506-3p on the proliferation of HUVECs, a CCK-8 assay was performed. The data indicated that the absorbance of HUVECs transfected with miR-506-3p inhibitor was significantly higher than that of HUVECs in the NC group 24 and 48 h after transfection (P<0.05; Fig. 3). By contrast, the absorbance of HUVECs transfected with miR-506-3p mimics did not differ from that of HUVECs in the NC group at 24 and 48 h (P>0.05; Fig. 3). These results indicate that the overexpression of miR-506-3p inhibits the proliferation of HUVECs.

Overexpression of miR-506-3p inhibits the migration ability of HUVECs. To determine the effect of miR-506-3p on the migration ability of HUVECs, a Transwell assay was conducted (Fig. 4). The data indicated that the number of HUVECs that passed through the chamber membrane in the miR-506-3p inhibitor group was significantly higher than in the NC group (P<0.05; Fig. 4B). However, the number of HUVECs did not differ significantly between the miR-506-3p mimics group and the NC group (P>0.05; Fig. 4B). These results suggest that the overexpression of miR-506-3p inhibits the migration ability of HUVECs.
miR-506-3p regulates the G1/S phases of HUVECs. To determine how miR-506-3p regulates the cell cycle of HUVECs, flow cytometry was used. The data indicated that the proportion of cells in G1 in the miR-506-3p inhibitor group (49.5±2.1%) was significantly lower than in the NC group (63.7±3.8%; P<0.05), whereas the proportion of cells in the S-phase in miR-506-3p inhibitor group (38.2±1.8%) was significantly higher than in the NC group (21.5±3.9%; P<0.05; Fig. 5). There were no differences in the proportion of cells in each phase of the cell cycle between the miR-506-3p mimics group and the NC group. These results indicate that miR-506-3p may suppress the proliferation of HUVECs by regulating the G1/S phase transition.

Overexpression of miR-506-3p promotes the apoptosis of HUVECs. To determine the effect of miR-506-3p on the apoptosis of HUVECs, flow cytometry was used (Fig. 6). The results demonstrated that the apoptotic rate of HUVECs transfected with miR-506-3p inhibitor was significantly lower than that of the NC group (P<0.05; Fig. 6B), whereas the apoptotic rate of HUVECs transfected with miR-506-3p mimics did not differ from that in the NC group (P>0.05; Fig. 6B). These results suggest that the overexpression of miR-506-3p promotes the apoptosis of HUVECs.

miR-506-3p exerts its biological role by regulating the expression of BECN1 protein. To determine the expression of BECN1, which is a target protein of miR-506-3p, western blotting was performed. The results demonstrated that levels of BECN1 in HUVECs transfected with miR-506-3p inhibitor were significantly higher than in the NC group (P<0.05; Fig. 7B), however the expression of BECN1 in HUVECs transfected with miR-506-3p mimics did not differ from that in the NC group (P>0.05; Fig. 7B). The result indicates that miR-506-3p may exert its biological role by regulating the expression of BECN1.

miR-506-3p regulates the expression of BECN1 by binding to the 3'-UTR of BECN1. To identify the interaction between miR-506-3p and BECN1, a dual-luciferase reporter assay was performed. The data demonstrated that transfection with
miR-506-3p mimics and pMIR-REPORT in the WT group led to significantly reduced fluorescence intensity compared with the NC (P<0.05). By contrast, fluorescence intensity in the mutant group did not differ significantly from that in the NC (P>0.05; Fig. 8). These results indicate that miR-506-3p regulates the expression of BECN1 by binding to the 3'-UTR of BECN1.

Discussion

Hypertension is a complex disease caused by the interaction between environmental and genetic factors. In recent years, studies have demonstrated that miRNAs are closely associated with the onset and development of hypertension (30,31). The expression of some miRNA molecules in vascular tissues is increased markedly. For example, miR-204 is highly expressed in vascular tissues and helps to determine blood pressure by regulating the proliferation and apoptosis of vascular smooth muscle cells (32,33). In addition, miR-155 regulates the expression of angiotensin II type 1 receptor, affects the relaxation and contraction of vascular endothelial cells and promotes the onset and development of hypertension (34). It has been demonstrated that miR-122 influences the expression of the amino acid transporter gene SLC7A by changing the small nucleotide polymorphism site, disrupting nitric oxide metabolism, regulating vascular endothelial cell function and promoting the onset of primary hypertension (35). The results of the current study demonstrate that miR-506-3p expression is upregulated in the peripheral blood of patients with hypertension and is dependent on the stage of hypertension.

It has been demonstrated that patients with hypertension develop vascular injury in the early course of the disease. Furthermore, vascular endothelial cell injury and dysfunction are important signs of hypertension and may even occur prior to the onset of hypertension (36). miRNAs serve important roles in endothelial cell injury and dysfunction. miR-34a promotes pulmonary arterial hypertension by regulating the proliferation and apoptosis of vascular endothelial cells (37). The in vitro experiments performed in the current study demonstrate that miR-506-3p inhibits the proliferation, G1/S phase transition and migration of HUVECs, and promotes the apoptosis of HUVECs.

BECN1 has three structural domains, Bcl-2-homology-3, the central coiled-coil domain and the evolutionarily conserved domain, by which it interacts with other molecules (38). BECN1 is essential for the formation of autophagosomes and regulates the formation and maturation of intracellular autophagosomes by mediating the localization of other autophagy proteins in phagophores (39). Elevated autophagy activity is associated with the upregulation of BECN1 expression and downregulating BECN1 expression inhibits autophagy activity (40). Additionally, it has been demonstrated that BECN1 is closely associated with tumorigenesis and organ damage (41,42).

In the current study, bioinformatics analysis indicated that BECN1 is a potential target gene of miR-506-3p. BECN1 is a homologous gene of autophagy-related 6 in yeast and regulates autophagy by forming a complex with phosphoinositide 3-kinase (38). Previous studies have demonstrated that autophagy is closely associated with vascular endothelial injury (43,44). The activation of autophagy contributes to the survival and proliferation of vascular endothelial cells under stress. In the present study, the results of the dual-luciferase reporter assay indicated that miR-506-3p directly regulates the expression of BECN1 and that BECN1 is a target gene of miR-506-3p.

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