MicroRNA-199a-5p aggravates primary hypertension by damaging vascular endothelial cells through inhibition of autophagy and promotion of apoptosis

XINTAO TIAN¹, CHUNPENG YU², LEI SHI¹, DAN LI³, XIAOXUE CHEN¹, DI XIA¹, JINGWEI ZHOU¹, WANQUN XU¹, CHENGTAI MA¹, LIHUA GU¹ and YI AN³

Departments of ¹Emergency Internal Medicine, ²Intervention and ³Cardiovascular Medicine, Laoshan District of The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266100, P.R. China

Received October 7, 2016; Accepted April 13, 2018

DOI: 10.3892/etm.2018.6252

Abstract. The present study investigated the expression of microRNA (miRNA or miR)-199a-5p in the peripheral blood of patients with primary hypertension, and examined its mechanism of action in vascular endothelial cell injury induced by hypertension. A total of 57 patients with primary hypertension, who were treated at the Affiliated Hospital of Qingdao University (Qingdao, China) between December 2014 and November 2015 were included in the present study. Peripheral blood was collected from all patients. The expression of miR-199a-5p was measured using reverse-transcription quantitative polymerase chain reaction analysis. Human umbilical vein endothelial cells (HUVECs) were divided into negative control, miR-199a-5p mimics and rescue (co-transfected with miR-199a-5p mimics and inhibitor) groups. After transfection, the proliferation and apoptosis of HUVECs were evaluated by a Cell Counting Kit-8 assay, a bromodeoxyuridine incorporation assay and flow cytometry. Western blot analysis was used to determine the expression of proteins involved in autophagy-associated and adenosine monophosphate kinase (AMPK)/unc-51 like autophagy activating kinase 1 (ULK1) signaling pathways. Laser scanning confocal microscopy and electron microscopy were used to observe the autophagy of HUVECs. The expression of miR-199a-5p was elevated in peripheral blood of patients with hypertension, and was correlated with the progression of hypertension. Overexpression of miR-199a-5p inhibited the proliferation and promoted the apoptosis of HUVECs. Upon expression of miR-199a-5p, the transition between microtubule-associated proteins 1A/1B light chain 3B (LC3B)I and LC3BII proteins was inhibited, the expression of p62 protein was upregulated. In addition, miR-199a-5p decreased the numbers of autophagosomes and autolysosomes in HUVECs. The present study demonstrated that expression of miR-199a-5p is positively correlated with the severity of hypertension. Expression of miR-199a-5p aggravated vascular endothelial injury by inhibiting autophagy and promoting the apoptosis of HUVECs via downregulation of the AMPK/ULK1 signaling pathway.

Introduction

Primary hypertension is a common chronic cardiovascular disease that causes cardiac and cerebrovascular accidents (1,2). The incidence of primary hypertension is increasing year by year, and the percentage of young patients (<20 years of age) affected is significantly elevated (3). Early prevention of primary hypertension and timely intervention of its development are of high significance in controlling the incidence of primary hypertension and alleviating the condition of affected patients. At present, the molecular mechanisms of hypertension remain to be fully elucidated. It has been reported that structural and functional abnormalities of blood vessels are early events in the occurrence and development of hypertension, including pathological processes, e.g. vascular endothelial cell injury, proliferation and migration of vascular smooth muscle cells, as well as vascular remodeling. Among these, vascular endothelial cell injury has an important implication in affecting vascular function in patients with hypertension (4). For instance, dysfunction of vascular endothelial cells in patients with hypertension interrupts the secretory homeostasis of nitric oxide and endothelin, induces vasoconstriction and increases circulation resistance, finally leading to the development of hypertension (5). Increased blood pressure promotes the apoptosis of microvascular endothelial cells, decreases parallel blood pathways and enhances peripheral circulation resistance, finally leading to hypertension (6). Although vascular endothelial cell injury induced by hypertension has an important role in the occurrence and development of hypertension, the molecular mechanisms remain to be fully elucidated.
Materials and methods

Patients. A total of 57 patients with primary hypertension, who were treated at the Affiliated Hospital of Qingdao University (Qingdao, China) between December 2014 and November 2015, were included in the present study. Peripheral blood was collected from all patients, as well as 31 age and gender-matched healthy subjects (control group), who were recruited during physical examination at the Affiliated Hospital of Qingdao University (Qingdao, China). The 57 patients included 34 males and 23 females, with an age range of 43-82 years, a mean age of 67.5 years and a median age of 64 years. The 57 patients were divided into three groups (I, II and III) according to the severity of hypertension determined according to the hypertension staging standard (14), with patients in group III having the most severe hypertension. The course of hypertension for each patient was >5 years.

Cells. Human umbilical vein endothelial cells (HUVECs; Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37°C and 5% CO₂. When reaching 70-90% confluence, the cells were seeded into 24-well plates at 1x10⁵ cells/well. At 48 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 PBS twice, followed by addition of DMEM and 10% FBS.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Peripheral blood (250 µl) was mixed with 0.75 ml TRIzol (Thermo Fisher Scientific, Inc.) for lysis, while the cells (1x10⁶) were trypsinized and mixed with 0.5 ml TRIzol for lysis. Total RNA was then extracted using the phenol chloroform method. The purity of the RNA was determined by measuring the ratio of the absorbance at 260 nm vs. that at 280 nm with an ultraviolet spectrophotometer (Nanodrop ND1000; Thermo Scientific, Inc.). Complementary (c)DNA was then obtained by RT of 0.5 µg RNA using the Reverse Transcription System (Takara Bio, Inc., Dalian, China) and stored at -20°C. The expression of miR-199a-5p was determined with the SYBR PrimeScript miRNA RT-PCR Kit (Takara Bio, Inc., USA) using U6 as an internal reference. The reaction system (20 µl) contained 10 µl ‘qPCR-mix, 0.5 µl upstream primer (5'-AGCTGTCTT AGCTGGTTG-3'), 0.5 µl downstream universal primer, 2 µl cDNA and 7 µl double-distilled H₂O. The reaction protocol was as follows: Initial denaturation at 95°C for 10 min; and 40 cycles of 95°C for 1 min and 60°C for 30 sec (iQ5; Bio-Rad Laboratories, Hercules, CA, USA). The 2⁻ΔΔCt method (15) was used to calculate the relative expression of miR-199a-5p against U6. Each sample was tested in triplicate.

Flow cytometry. At 48 h after transfection, HUVECs were trypsinized, collected and washed with pre-cooled PBS twice. The apoptosis of cells was examined by flow cytometry using BD Pharmingen FITC Annexin V Apoptosis Detection kit I (cat. no. 556547; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Early apoptotic cells were identified by positive staining with Annexin V only, necrotic cells were indicated by positive staining with propidium iodide only, and late apoptotic cells were identified by double-positive staining with propidium iodide and Annexin V. In order to observe whether autophagy regulates apoptosis in HUVECs, the cells were incubated with autophagy inhibitor 3-methyladenine (3-MA; 50 nM) for 24 h prior to flow cytometric analysis.
Western blot analysis. HUVECs were trypsinized, collected and suspended with 100 µl pre-cooled radiolmounoprecipitation assay lysis buffer containing 1 mM phenylmethylsulfonyl fluoride for lysis at 4°C for 15 min. Subsequently, the cells were centrifuged at 12,000 g for 5 min. The supernatant was used to determine protein concentration (4 µg/µl), using a BCA protein concentration determination kit (cat. no. RTP7102; Real-Times Biotechnology Co., Ltd., Beijing, China). The samples were then mixed with 5x loading buffer. After denaturation in a boiling water bath for 10 min, the samples (20 µg) were subjected to 10% SDS-PAGE at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.) on ice (300 mA, 1 h) and blocked with 5% skimmed milk in PBS containing Tween-20 at room temperature for 1 h. Subsequently, the membranes were incubated with rabbit anti-human polyclonal primary antibodies to light chain (LC)3B (1:1,000 dilution; cat. no. ab51520); p62 (1:1,000 dilution; cat. no. ab91526) and GAPDH (1:5,000 dilution; cat. no. ab8245; Abcam, Cambridge, UK) at 4°C overnight. After extensive washing with PBS containing Tween-20 for 6 times (5 min each), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibody (1:8,000 dilution; cat. no. ab6721; Abcam) for 1 h at room temperature prior to washing with PBS containing Tween-20 for 6 times (5 min each). Subsequently, the membranes were developed with an enhanced chemiluminescence detection kit (Sigma-Aldrich; Merck KGaA) for imaging. Image lab v3.0 software (Bio-Rad Laboratories) was used to acquire and analyze imaging signals. Relative protein contents were quantified against GAPDH.

Electron microscopy. At 48 h after transfection, HUVECs were trypsinized and centrifuged, followed by re-suspension with PBS. After discarding the supernatant, the cells were made into single suspension and then fixed with 2.5% glutaraldehyde at 4°C overnight. After washing with PBS once, the cells were fixed with 1% osmic acid for 1 h. After preparation of slides, autophagosomes were observed under an electron microscope (JEM1230; JEOL, Tokyo, Japan).

Laser scanning confocal microscopy. HUVECs (1x10⁵) were seeded onto specialized cell culture dishes for laser scanning confocal microscopy. At 24 h after transfection, the HUVECs were transfected with Ad-green fluorescent protein-red fluorescent rotein-LC3 adenovirus (multiplicity of infection, 20; cat. no. HB-AP210 0001; HanBio Biotechnology Co., Ltd.). At 12 h after transfection, the medium was replenished. At 72 h, the cells in each group were fixed with 4% polyoxymethylene and washed with PBS. The cells were observed under an SP8 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Green vesicles represented autophagosomes and red vesicles represented autolysosomes. Cells in 5 fields were individually counted to obtain a mean value.

Statistical analysis. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as the mean ± standard deviation. Data were compared between two groups by using a Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-199a-5p in peripheral blood is positively associated with the progression of hypertension. To measure the expression of miR-199a-5p in peripheral blood of patients with hypertension, RT-qPCR was employed. The results indicated that the level of miR-199a-5p in peripheral blood from patients with primary hypertension were significantly higher than that in healthy subjects (P<0.05; Fig. 1A). In addition, the serum levels of miR-199a-5p in patients with grade I or II hypertension were significantly lower than those from patients with grade III hypertension (P<0.05), while they were not significantly different between patients with grade I and those with grade II hypertension (P>0.05; Fig. 1B). These results suggest that the expression of miR-199a-5p in peripheral blood is associated with the progression of hypertension.

Overexpression of miR-199a-5p inhibits the proliferation of HUVECs. To assess the proliferation of HUVECs, a CCK-8 assay was performed. The results indicated that the number of HUVECs in the group transfected with miR-199a-5p mimics was significantly lower than that in the NC group at 48 and 72 h (P<0.05). In addition, the number of HUVECs in the rescue group was significantly higher than that in the miR-199a-5p mimics group at 48 and 72 h (P<0.05), but was not different from that in the NC group (P>0.05; Fig. 2). The result indicates that overexpression of miR-199a-5p inhibits the proliferation of HUVECs.

Overexpression of miR-199a-5p promotes the apoptosis of HUVECs. To detect apoptosis of HUVECs, a flow cytometric assay was performed. In the NC group, the percentages of necrotic cells, as well as cells in the early and late stage of apoptosis were 1.86, 1.47 and 4.27%, respectively, while they were 2.10, 8.80 and 18.50%, respectively, in the miR-199a-5p group. In the rescue group, the percentages were 1.30, 6.50 and 10.80%, respectively (Fig. 3A). Quantification of the percentage of total apoptotic cells (early stage from lower right quadrant and late stage from upper right quadrant) indicated that the apoptotic rate of HUVECs transfected with miR-199a-5p mimics was significantly higher than that in the NC group (P<0.05). Furthermore, the apoptotic rate of HUVECs in the rescue group was significantly lower than that in the miR-199a-5p mimics group (P<0.05), but significantly higher than that in the NC group (P<0.05; Fig. 3A and B). These results suggest that overexpression of miR-199a-5p promotes the apoptosis of HUVECs.

miR-199a-5p aggravates vascular endothelial injury by inhibiting autophagy and promoting apoptosis. To examine the effect of miR-199a-5p overexpression on autophagy of HUVECs, laser scanning confocal microscopy, western blot analysis, electron microscopy and flow cytometry were utilized. Confocal microscopy indicated that the numbers of autophagosomes and autolysosomes in HUVECs transfected with miR-199a-5p mimics were significantly lower than those in the NC group (P<0.05). In addition, the numbers of autophagosomes and autolysosomes in HUVECs of the rescue group were significantly higher than those in the miR-199a-5p mimics group (P<0.05). In addition, the number of autophagosomes...
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...results suggest that miR-199a-5p aggravates vascular endothelial injury by inhibiting autophagy and inducing apoptosis, and that the downregulation of autophagy itself promotes HUVEC apoptosis.

miR-199a-5p inhibits autophagy and promotes apoptosis of HUVECs via the adenosine monophosphate kinase (AMPK)/unc‑51 like autophagy activating kinase 1 (ULK1) signaling pathway. To determine the effect of miR-199a-5p expression on the AMPK/ULK1 signaling pathway, western blot analysis, flow cytometry and laser scanning confocal microscopy were performed. Western blot analysis indicated that the phosphorylation levels of AMPK-α, acetyl-CoA carboxylase (ACC) and ULK1 in the miR-199a-5p mimics group were reduced compared with those in the NC or rescue group (data not shown). In addition, treatment with metformin enhanced the phosphorylation levels of AMPK-α, ACC and ULK1 compared with those in the miR-199a-5p mimics group (data not shown). Flow cytometry indicated that metformin treatment inhibited apoptosis of HUVECs transfected with miR-199a mimics (Fig. 8). In addition, confocal microscopy demonstrated that metformin treatment activated autophagy of HUVECs transfected with miR-199a mimics (Fig. 9). These results suggest that miR-199a-5p inhibits autophagy and promotes apoptosis of HUVECs via the AMPK/ULK1 signaling pathway.

Discussion

Primary hypertension is a common chronic cardiovascular disease that causes vascular endothelial cell injury and inflammation in patients, leading to various complications (16). Early diagnosis and timely intervention of vascular endothelial injury have great significance in the prevention and delay of damage to tissues and organs. In the development of hypertension and its complications, the expression of multiple miRNA molecules is changed, suggesting their clinical and diagnostic value for injuries of the heart, brain and blood vessels (17). In the present study, the expression of miR-199a-5p in the peripheral blood of patients with primary hypertension was indicated to be elevated, and miR-199a-5p was demonstrated to inhibit...
Figure 3. Effect of miR-199a-5p on the apoptosis of HUVECs. (A) Flow cytometry plots of HUVECs. (B) Apoptotic rate of HUVECs. Apoptotic cells at early (lower right quadrant) and late (upper right quadrant) stages were included for quantification. *P<0.05 compared with NC group; #P<0.05 compared with miR-199a-5p mimics group. HUVECs, human umbilical vein endothelial cells; miR, microRNA; NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Figure 4. Effect of miR-199a-5p mimics on the number of autophagy vesicles in HUVECs. (A) Confocal microscopic images of HUVECs obtained by laser scanning confocal microscopy (magnification, ×400). Green vesicles represent autophagosomes and red vesicles represented autolysosomes. (B) Numbers of autophagosomes and autolysosomes in HUVECs counted in five fields of view. *P<0.05 compared with NC group; #P<0.05 compared with miR-199a-5p mimics group. HUVECs, human umbilical vein endothelial cells; miR, microRNA; NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide; GFP, green fluorescence protein; RFP, red fluorescence protein; merge, fusion of GFP and RFP images.

Figure 5. Effect of miR-199a-5p on the expression of autophagy-associated proteins. (A) Western blots of autophagy-associated proteins. (B) Ratio of LC3BII protein expression against LC3BI protein expression. (C) Relative expression of p62 protein. *P<0.05 compared with NC group; #P<0.05 compared with miR-199a-5p mimics group. LC3B, microtubule-associated proteins 1A/1B light chain 3B; miR, microRNA; NC, negative control.
autophagy of HUVECs by downregulating the activity of the AMPK/ULK1 signaling pathway, finally leading to damage of vascular endothelial cells.

Vascular endothelial cells are important barriers in the vascular lumen. They not only maintain the stability of hemodynamics and material exchange, but also secrete inflammatory cytokines, as well as vascular relaxation and contraction factors, which regulate blood pressure. Abnormal structure and function of vascular endothelial cells are important factors that promote the development of hypertension (18). Persistent hypertension may cause injury of vascular endothelial cells.
cells, further influencing the relaxation and contraction of blood vessels, and facilitating the development of hypertension. It has been reported that various miRNAs have important roles in the injury of vascular endothelial cells in normal or tumor tissues. For instance, miR-129-1 and miR-133 have been demonstrated to promote the proliferation of vascular endothelial cells (19). In addition, miR-506 inhibits tumor angiogenesis in hepatocellular carcinoma (20). miR-141 reduces the expression of intercellular adhesion molecule-1 in endothelial cells, and alleviates myocardial ischemia/reperfusion injury (21). The newly discovered miR-199a-5p has been reported to inhibit the proliferation, invasion and migration of various types of tumor cells, and its expression was downregulated in certain tumor types (13). Reduced expression of miR-199a-5p is associated with methylation of its promoter (22). In cardiovascular diseases, downregulation of miR-199a expression is closely associated with the hypertrophy of cardiac muscle cells (23). The results of the present study indicate that miR-199a-5p expression is upregulated in patients with hypertension, and that miR-199a-5p mimics inhibit the proliferation and promote the apoptosis of HUVECs. Furthermore, miR-199a-5p expression is also increased in smooth muscle cells and promotes hypertension. For instance, Hashemi Gheinani et al. (24) reported that miR-199a-5p regulates smooth muscle cell proliferation and morphology by targeting the WNT2 signaling pathway. Liu et al. (25) confirmed that miR-199a-5p was increased in human pulmonary artery smooth muscle cells, and that it was associated with pulmonary artery hypertension. The results of the present study reveal that miR-199a-5p expression in HUVECs is decreased compared with that in human vascular smooth muscle cells.

Autophagy is an important biological process that is evolutionarily conserved in eukaryotic cells and maintains homeostasis of circulating intracellular substances. It has a variety of roles in inflammation, immunity and tumors. For instance, the nucleotide-binding oligomerization domain-containing protein 2 gene controls bacterial infection of intestinal epithelial cells by regulating autophagy (26), and nuclear factor (erythroid-derived 2)-like 2 participates in acute lung injury by regulating autophagic activity (27). Under stress conditions, autophagy is significantly increased and this process is dependent on the regulation of a series of autophagy-associated genes. It has been reported that miR-183, miR-376b and miR-106a participate in the regulation of autophagy. In addition, Li et al. (28) reported that miR-199a-5p inhibits autophagy via the mammalian target of rapamycin signaling pathway, finally leading to cardiomyocyte hypertrophy. Xu et al. (29) discovered that downregulation of miR-199a-5p expression in hepatocellular carcinoma activates autophagy of tumor cells, and enhances resistance to chemotherapy. The present study reported that miR-199a-5p inhibits AMPK signaling in vascular endothelial cells. It is known that activation of the AMPK signaling pathway leads to the phosphorylation of ULK1 and the activation of autophagy (30). In addition, treatment with metformin, an AMPK activator, inhibited apoptosis and enhanced autophagy of HUVECs induced by miR-199a-5p. These results suggest that miR-199a-5p aggravates vascular endothelial injury by inhibiting autophagy and promoting apoptosis via the suppression of the AMPK signaling pathway and inhibition of ULK1 phosphorylation.

In conclusion, the present study demonstrates that miR-199a-5p expression is enhanced in peripheral blood of patients with hypertension. miR-199a-5p regulates the AMPK signaling pathway, inhibits autophagy and promotes vascular endothelial injury induced by hypertension, and may be a suitable marker for the clinical diagnosis as well as a target for the treatment of hypertension.

Acknowledgements
The authors would like to thank the Affiliated Hospital of Qingdao University (Shandong, China) for their support.

Funding
No funding was received.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
XT, CY and YA designed the study; XT, CY, LS, DL, XC, DX, JZ, WX, CM and LG performed experiments; XT, CY, DL and YA analyzed the data. The final version of the manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work.

Ethical approval and consent to participate
All procedures were approved by the Ethics Committee of Qingdao University (Qingdao, China). Written informed consent was obtained from all patients or their families.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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