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Analysis of Abnormal Expression of MiR-320b in Serum of Patients with Hypertension and its Clinical Value

Xiaoyan Wang,[1] Hongxia Gong,[2] Xuhua Li[1] and Xiaofang Chen[1]

[1]Department of Internal Medicine, Zhejiang Normal University Hospital, Jinhua, Zhejijang, China
[2]Department of Cardiology, Yiwu Central Hospital, Yiwu, Zhejijang, China

Studies have found that miRNAs can participate in the progression of hypertension by affecting the function of endothelial cells and inflammatory response. This study was to investigate the clinical value of miR-320b in patients with hypertension and its potential effect on Angiotensin (Ang) II-induced endothelial cells. Real-time quantitative PCR (RT-qPCR) was used to detect the differential expression of miR-320b in all subjects, and the diagnostic value of miR-320b in hypertension was further evaluated by the receiver operating characteristic (ROC) curve. Ang II-induced human umbilical vein endothelial cells (HUVECs) were established as a model of hypertension injury. The possible downstream target gene AKT serine/threonine kinase 3 (*AKT*) of miR-320b was predicted through TargetScan, and the interaction between miR-320b and *AKT3* was verified by luciferase reporter gene. The results showed that serum miR-320b was reduced in patients with hypertension compared with healthy people ($P < 0.001$). With the increase of hypertension grade, the serum miR-320b level of patients gradually decreased ($P < 0.001$). ROC analysis showed that miR-320b had the ability to distinguish patients from healthy people. Cell analysis proved that Ang II induced the decrease of HUVECs viability and the activation of apoptosis and inflammation, while overexpression of miR-320b inhibited Ang II-induced apoptosis and inflammation and promoted cell growth ($P < 0.05$). Luciferase reporter gene showed that *AKT3* was the downstream target gene of miR-320b. In summary, this study suggests that miR-320b alleviates Ang II-induced apoptosis, inflammation and the inhibition of cell viability by targeting *AKT3* expression, and may be involved in the pathogenesis of hypertension.

Keywords: AKT3; hypertension; inflammation; miR-320b
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Introduction

With the improvement of living standards and the change of diet structure, the incidence of hypertension has been increasing year by year, which has become a major public health problem threatening human health. Hypertension can be divided into two major categories: primary hypertension and secondary hypertension (Chen et al. 2017), and primary hypertension accounts for more than 95% of the total patients with hypertension (Asla et al. 2022). Hypertension is a disease caused by the interaction of environmental factors and genetic factors, and is a risk factor for cardiovascular and cerebrovascular diseases (Gao et al. 2017). Long-term persistent increase in blood pressure will lead to changes in the structure of the heart and blood vessels, and the typical pathological changes are left ventricular hypertrophy and myocardial fibrosis, which seriously affect the patients' physical and mental health (Diez 2008; Fan et al. 2022). For a long time, researchers at home and abroad have been committed to overcoming hypertension from the mechanism, although much progress has been made, hypertension is still incurable so far. With the deepening of research and the continuous study of genetic factors, microRNAs (miRNAs) have become a new hotspot in the study of hypertension.

MiRNA is a class of endogenous non-coding and highly conserved small single-stranded RNA, which is involved in the expression and transcription of many genes in organisms (Lee et al. 2014). MiRNA plays an important role in regulating biological processes such as cardiac

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Correspondence: Xiaoyan Wang, Department of Internal Medicine, Zhejiang Normal University Hospital, No. 688, Yingbin Avenue, Jinhua 321001, China.
e-mail: wangxiaoyandr0706@163.com

development, angiogenesis, myocardial hypertrophy, and myocardial fibrosis (Kriegel et al. 2015; Solayman et al. 2016). Studies have shown that miR-31 promotes the development of myocardial fibrosis by regulating epithelial-mesenchymal transformation in epicardial mesothelial cells (Moskalik et al. 2022). Other studies have shown that miR-499 can cause myocardial hypertrophy in hypertensive mice (Corsten et al. 2010). The miR-320 family includes miR-320-3p, miR-320b, and miR-320c, all located on chromosome 8, which are closely related to the occurrence of cancer (Xu et al. 2023). In recent years, miR-320b has been found to be closely related to the occurrence of cardiovascular and cerebrovascular diseases. Zhang et al. (2016) reported the decrease of serum miR-320b in patients with carotid atherosclerosis, which is a specific serum marker for carotid atherosclerosis and vulnerable plaque. In a study of obese people, it was observed that eight miRNAs, including miR-320b, were significantly reduced in obese people and negatively correlated with visceral fat content (Choi et al. 2020). At present, although miR-320b has been reported in cardiovascular diseases, the expression of miR-320b in hypertension and its effect on vascular endothelial cells have not been reported yet. The purpose of this study aims to investigate the changes in miR-320b expression in the blood of patients with hypertension, and to further reveal its regulatory mechanism in Ang II-induced vascular endothelial cells.

Materials and Methods

Study population

Fifty-nine patients diagnosed with essential hypertension in Zhejiang Normal University Hospital were recruited as study group, and 62 healthy adults from the physical examination department of this hospital were selected as the healthy control group. The selection criteria of subjects: (1) They are over 18 years old and meet the diagnostic criteria of essential hypertension (Joint Committee for Guideline Revision 2019); (2) Complete clinical data; (3) Newly diagnosed or previously diagnosed but not receiving standardized antihypertensive drugs. Exclusion criteria: (1) Patients with secondary hypertension or those who are taking antihypertensive drugs; (2) Patients with diabetes, hyperlipidemia, stroke, heart failure, cardiomyopathy, and atrial fibrillation; (3) Patients with liver and kidney failure; (4) Patients with malignant tumor, infectious disease, and autoimmune disease. This study was approved by the Ethics Committee of Zhejiang Normal University Hospital, and all subjects gave their informed consent. All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki Declaration and later versions.

Collection of blood samples

Ten mL of fasting venous blood was collected to detect blood biochemical indexes and the level of miR-320b. The whole blood was used for the determination of blood routine indicators, and the serum was used for the detection of miR-320b expression. The separation of serum was obtained based on previously published literature (Yao et al. 2023). In short, the venous blood was placed in a centrifuge tube without anticoagulant at room temperature for 30 min, followed by centrifugation at 3,500 rpm for 15 min, and the upper serum was stored in a refrigerator at −80°C for future use.

Measurement of blood pressure

All subjects had their blood pressure measured by medical mercury sphygmomanometer confirming to the international measurement standards (Esmaillzadeh et al. 2015). Thrity minutes before the measurement, all subjects were asked to abstain from smoking, alcohol, and coffee. Empty the bladder and remain quiet for at least 5 min before measurement. The measured person takes a sitting position so that the upper arm is at the same level as the heart. During the measurement, the gasbag needs to be inflated quickly, and the Korotkoff sound of the mercury column should be heard during the deflation of the gasbag. At the same time, the vertical height of the mercury column should be observed and recorded when the Korotkoff sound appears and disappears. The systolic blood pressure (SBP) is the reading of mercury column when the Korotkoff sound appears, and diastolic blood pressure (DBP) is the reading when the Korotkoff sound disappears. The measurement was repeated after an interval of 5 minutes, and the average value of the two measurements was taken.

Cell culture and model establishment

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. HUVECs are suitable for cultivation in a constant temperature incubator containing 5% CO_2 at 37°C. The culture medium was changed once a day. When the cells grew to cover about 70%-80%, they were digestion and passage with pancreatic enzyme, and 1×10^5 cells/well were inoculated into the 96-well plate for subsequent experiments. According to the previously published literature, angiotensin (Ang) II-induced culture medium was used to culture HUVECs to construct the injury model (Xu et al. 2021b).

Cell transfection and grouping

In this experiment, miR-320b mimic and mimic-negative control (NC) were designed and synthesized by GenePharm and transfected into HUVECs according to the instructions of Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). An appropriate amount of HUVECs was inoculated into the 6-well plate the day before the experiment to ensure that the cell fusion was above 80% at the beginning of transfection. It was calculated that 8 μL of Lipofectamine

2000 (Invitrogen) and 500 pmol of miR-320b mimic should be added to each well. The evenly mixed and incubated transfer solution was added to HUVECs cultured with serum-free culture medium, and the cells were placed in the incubator for 6 h. After transfection, the liquid in the plate was discarded, and the transfer solution was washed with sterile phosphate-buffered saline (PBS). The culture medium containing 1 μmol/L Ang II continued to be cultured for 48 h. Cell groups consisted of control group, Ang II group, Ang II + miR-NC group, Ang II + miR-320b mimic group.

Real-time fluorescence quantitative PCR

The total RNA was extracted with TRIzol reagent, a 1 μL RNA sample was taken for the determination of concentration, and the absorbance ratio of the RNA sample (A260/A280) was calculated. When the ratio is between 1.8 to 2.0, it indicated that the RNA sample was of high purity. The reverse transcription from RNA to cDNA was completed according to the instruction of Primescript RT kit. Subsequently, a PCR reaction was performed using SYBR Premix Ex Taq kit. The reaction system was 20 μL containing 10 μL kit, 0.5 μL upstream primer, 0.5 μL downstream primer, 2 μL cDNA template, and 7 μL double distilled water. The amplification procedures were as follows: predenaturation at 95°C for 5min, denaturation at 95°C for 5 seconds, annealing at 60°C for 40 seconds, extension at 72°C for 25 seconds, all 40 cycles. Using U6 as the internal reference, the relative expression level of miR-320b was calculated by $2^{-\triangle\triangle Ct}$ method. The sequence of primers required for the experiment is as follows: miR-320b, forward primer 5'-GATGCTGAAAAGCTGGGTT-3'; reverse primer 5'-TATGGTTGTTCTGCTCTCTG-3': U6, forward primer 5'-GCTTCGGCAGCACATATACTAAAAT-3'; reverse primer 5'-CGCTTCACGAATTTGCGTGTCAT-3'.

Cell viability

The cell viability was detected by Cell Counting Kit-8 (CCK-8) assay. The specific steps were as follows: HUVECs were inoculated into 96-well plates at a density of 2×10^3 cells per 100 μL cell suspension. After the cell fusion reached more than 80%, cell transfection was performed first, followed by Ang II induction. After incubation, at every preset time point, the old culture medium was sucked out and 100 μL serum-free medium containing 10 μL CCK-8 solution was added to each well and continued to be incubated for 2 h. Finally, the absorbance of each well at 450 nm was measured by microplate reader, and the cell viability was calculated.

Cell apoptosis

Flow cytometry was used to detect cell apoptosis. The steps were as follows: HUVECs were inoculated into 6-well plates, and when the cell adhesion growth and fusion reached more than 80%, cell transfection was performed first, followed by Ang II induction. After treating the cells according to the experimental procedure, pancreatic enzymes were added to digest the cells and the cells were fully suspended. After centrifugation, the cells were washed thoroughly twice with pre-cooled PBS. The supernatant was discarded, 300 μL binding buffer was added to re-suspend the cells, and then 5 μL Annexin-V/FITC and 5 μL propidium iodide (PI) dye were added successively. The cell suspension was gently shaken and reacted at room temperature for 15 min in the dark. Finally, make sure that the flow cytometry test is performed within 1 h.

Enzyme-linked immunosorbent assay (ELISA)

Cells were inoculated into 12-well plates and treated according to experimental requirements. The cell supernatant was collected and the levels of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in the cell supernatant were measured according to the instructions of the commercially available ELISA kit.

Luciferase reporter gene assay

TargetScan online database was used to predict the target gene of miR-320b, and literature review showed that AKT serine/threonine kinase 3 (AKT) was closely related to the occurrence and development of hypertension (Li et al. 2020). The conservative site sequences of miR-320b target gene *AKT3* with the lowest free energy were selected, and the wild-type and mutant *AKT3* seed regions were synthesized *in vitro* by chemical synthesis. The *AKT3* fragment containing has-miR-320b binding site was cloned into the plasmid pMIR vector, and pMIR-ATK3-3'-UTR-WT was constructed. In addition, pMIR-ATK3-3'-UTR-MUT reporter vector containing mutant target gene loci was constructed. The construction of these vectors was completed by MedChemExpress (Guangzhou, China). Lipofectamine 2000 (Invitrogen) was used to co-transfect these vectors with miR-320b mimic or inhibitor into HUVECs. After 48 h of transfection, the luciferase activity was measured using a dual-luciferase reporter gene assay (Promega Corporation, Madison, WI, USA). The sequences used in this study were as follows: miR-320b mimic, AAAAGCUGGGUUGAGAGGGCAA; mimic-NC, UUCUCCGAACGUGUCACGUTT; miR-320b inhibitor, UUCUCCGAACGUGUCACGUTT, inhibitor-NC, CAGUACUUUUGUGUAGUACAA.

Data analysis

SPSS 20.0 software was used for statistical analysis of all the data collected in this study. The Komlogorov-Smirnov test was used to determine the normality of the data, and the data conforming to the normal distribution was expressed as the mean ± standard deviation (SD). Independent sample t test was used for the comparison between two groups, and one-way analysis of variance (ANOVA) was used for the comparison in multiple groups. The sample size required in this study was calculated using Power Analysis and Sample Size version 11.0 software (PASS 11.0, NCSS, Kaysville, UT, USA) based on the significance at 5% (α = 0.05, two sided), power of 90% (β =

0.10), and 10% dropout rate. At least 50 subjects should be included in each group. Receiver operating characteristic (ROC) curve was conducted to evaluate the accuracy of miR-320b in clinical diagnosis of hypertension, and Pearson correlation coefficient was carried out to assess the correlation between miR-320b and clinical indicators. All experiments are in triplicate. $P < 0.05$ was considered statistically significant.

Results

Comparison of baseline data and clinical information between the two groups

The baseline data and clinical indicators of the healthy control group and the hypertension group are summarized in Table 1. The results showed that there were significant differences in SBP, DBP, total cholesterol (TC), and serum creatinine (Scr) between the two groups ($P < 0.001$). Additionally, no significant differences in sex, age, body mass index (BMI), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), fasting blood-glucose (FBG), and alanine transaminase (ALT) were observed between the two groups ($P > 0.05$).

The expression level of serum miR-320b and its clinical diagnostic value in hypertension

The level of serum miR-320b was detected by RT-qPCR. The results showed that the serum level of miR-320b in patients with hypertension was significantly upregulated compared with that in healthy people (Fig. 1A, $P < 0.001$), which suggested that the abnormal expression of miR-320b may be involved in the pathogenesis of hypertension. Besides, according to the classification of hypertension, it was found that the level of miR-320b decreased with the increase of the classification, and it was preliminarily determined that the expression of miR-320b was associated with the severity of hypertension (Fig. 1B, $P < 0.001$). Subsequently, the clinical diagnostic significance of miR-320b in hypertension was evaluated by ROC analysis. As shown in Fig. 2, the area under the curve (AUC) value of the ROC curve is 0.896, and the sensitivity and specificity are 88.7% and 79.7% respectively at the cut-off value of 0.7663, suggesting that miR-320b has high clinical diagnostic accuracy for hypertension.

Correlation analysis between miR-320b level and clinical indicators

Pearson correlation coefficient was applied to estimate the correlation between miR-320b expression level and clinical indicators in patients with hypertension. Table 2 results showed that the levels of SBP ($r = -0.673$, $P < 0.001$), DBP ($r = -0.564$, $P < 0.001$), LDL-C ($r = -0.532$, $P < 0.001$) and TC ($r = -0.181$, $P < 0.05$) in patients with hypertension are significantly negatively correlated with miR-320b level.

Effects of miR-320b on the viability and apoptosis of HUVECs cells induced by Ang II

The expression level of intracellular miR-320b was regulated by cell transfection. After transfection of miR-

Table 1. Basic clinical information of the subject.

Clinical indicators	Healthy controls (n = 62)	Hypertension patients (n = 59)	*P*
Sex (female/male)	29/33	26/33	0.106
Age (Years)	58.52 ± 7.91	57.94 ± 8.03	0.433
BMI (kg/m^2)	22.41 ± 3.23	22.13 ± 3.15	0.874
SBP (mmHg)	123.61 ± 11.02	169.52 ± 10.38	< 0.001
DBP (mmHg)	78.37 ± 6.52	111.54 ± 12.32	< 0.001
TC (mmol/L)	4.37 ± 0.76	4.73 ± 1.18	< 0.001
TG (mmol/L)	1.62 ± 0.85	1.83 ± 0.95	0.158
HDL-C (mmol/L)	1.35 ± 0.23	1.36 ± 0.34	0.433
LDL-C (mmol/L)	2.88 ± 1.01	3.01 ± 0.69	0.330
FBG (mmol/L)	5.21 ± 0.83	5.32 ± 0.59	0.071
Scr (μmol/L)	65.28 ± 12.73	72.42 ± 20.91	0.001
ALT (U/L)	24.29 ± 13.03	24.34 ± 13.50	0.672
Hypertension classification			
I (n, %)	/	8 (13.56%)	/
II (n, %)	/	39 (66.10%)	/
III (n, %)	/	12 (20.34%)	/

Data are expressed as n or mean ± standard deviation (SD). $P < 0.05$ was a significant difference.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; FBG, fasting blood-glucose; Scr, serum creatinine; ALT, alanine transaminase.

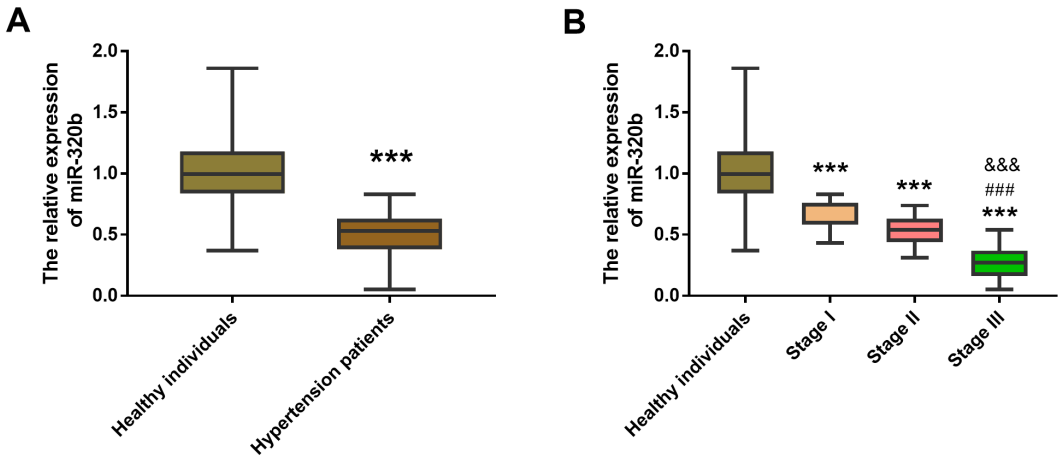

Fig. 1. The expression level of serum miR-320b in all individuals.
(A) RT-qPCR results showed that the expression of serum miR-320b was decreased in patients with hypertension. (B) The expression of miR-320b decreased gradually with the severity of hypertension. $^{***}P < 0.001$ vs. healthy people; $^{###}P < 0.001$ vs. Stage I; $^{\&\&\&}P < 0.001$ vs. Stage II.

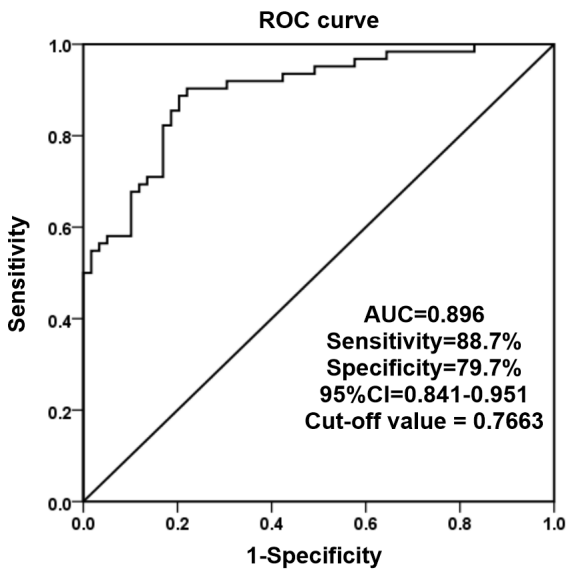

Fig. 2. Receiver operating characteristic (ROC) curve showed that serum miR-320b had the ability to distinguish between patients with hypertension and healthy people (AUC: 0.896, sensitivity: 88.7%, specificity: 79.7%, cut-off value: 0.7663).

Table 2. Correlation between miR-320b and some clinical indicators.

Clinical indicators	r	*P*
SBP (mmHg)	−0.673	< 0.001
DBP (mmHg)	−0.564	< 0.001
TC (mmol/L)	−0.181	0.049
TG (mmol/L)	−0.111	0.224
HDL-C (mmol/L)	0.179	0.050
LDL-C (mmol/L)	−0.532	< 0.001
FBG (mmol/L)	0.066	0.469
Scr (μmol/L)	−0.119	0.293
ALT (U/L)	0.061	0.508

The correlation coefficient (r) is presented. $P < 0.05$ was a significant difference.
SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; FBG, fasting blood-glucose; Scr, serum creatinine; ALT, alanine transaminase.

320b mimics, the expression level of miR-320b in the control group was significantly improved (Fig. 3A, $P < 0.001$). As shown in Fig. 3B, compared with the control group, the level of miR-320b in HUVECs induced by Ang II was significantly down-regulated, indicating that the establishment of *in vitro* hypertensive cell model was successful ($P < 0.001$). Besides, the intracellular level of miR-320b increased directly after transfecting the miR-320b mimic ($P < 0.001$). CCK-8 experiment revealed that the cell viability of HUVECs was inhibited after induction with Ang II, which indicated that the presence of Ang II had an inhibitory effect on the growth of HUVECs. After upregulating the level of miR-320b, the cell viability was significantly improved (Fig. 3C, $P < 0.001$). Flow cytometry indicated that the number of apoptotic cells increased significantly after Ang II stimulation, while the apoptotic rate of cells transfected with miR-320b mimic decreased significantly, which suggested that miR-320b could inhibit Ang II-induced cell apoptosis (Fig. 3D, $P < 0.001$).

Effects of miR-320b on the inflammatory response of HUVECs cells induced by Ang II

The concentration of inflammatory cytokines in the cell supernatant was determined by ELISA. The results showed that the production of IL-1β, IL-8 and TNF-α in

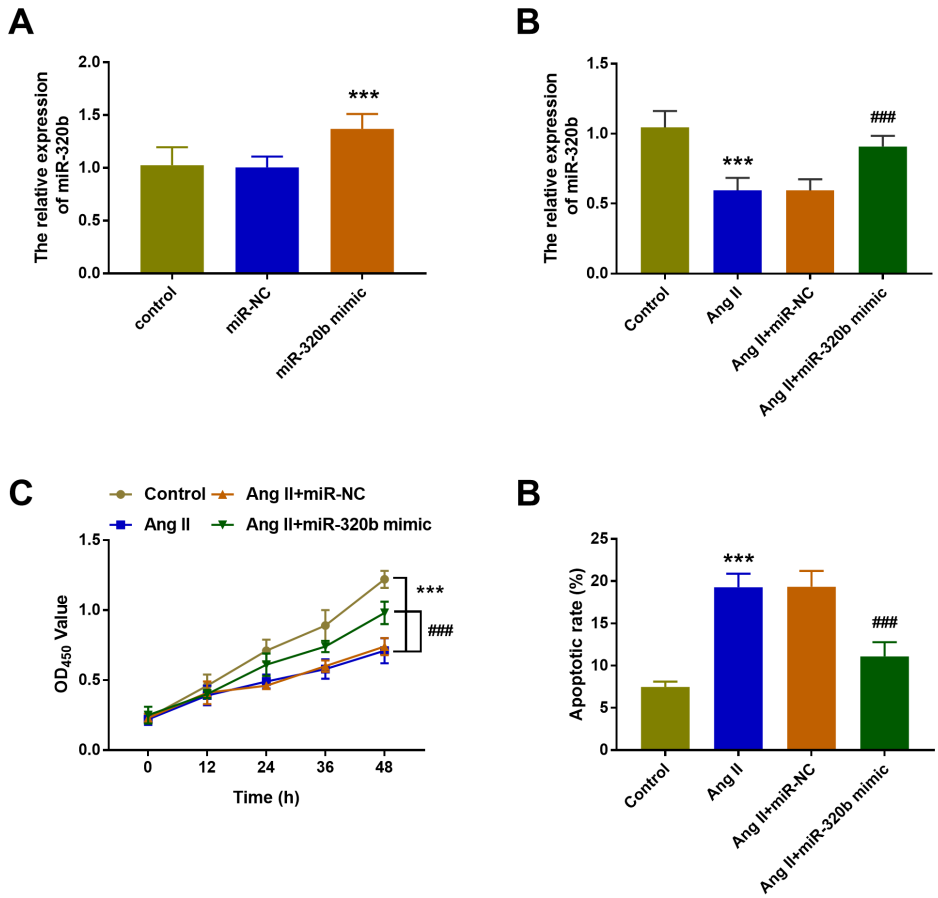

Fig. 3. Effects of miR-320b on the viability and apoptosis of human umbilical vein endothelial cells (HUVECs) induced by angiotensin (Ang) II.
(A) Cell transfection regulates the expression of miR-320b in HUVECs (n = 3 per group). (B) Cell transfection regulates the expression of miR-320b in Ang II-induced HUVECs (n = 3 per group). (C) The effect of miR-320b on the viability of hypertensive cell models was detected by CCK-8 assay (n = 6 per group). (D) The effect of miR-320b on the cell apoptosis of hypertensive cell models was detected by flow cytometry (n = 3 per group). $^{***}P < 0.001$ vs. control group; $^{###}P < 0.001$ vs. Ang II group.

HUVECs increased significantly after being induced by Ang II, indicating that Ang II activated the inflammatory response. However, after the upregulation of miR-320b, the concentrations of the above inflammatory factors decreased in different degrees, which indicated that the increase of miR-320b was helpful to inhibit the inflammatory response induced by Ang II (Fig. 4A-C, $P < 0.05$).

Validation of targeting relationship between miR-320b and AKT3

In order to further evaluate the mechanism of miR-320b in regulating cell function and inflammation in HUVECs, we predicted the possible target genes of miR-320b using TargetScan database, and finally found that miR-320b and AKT3 have complementary binding sites (shown in Fig. 5A). Then, the interaction between miR-320b and AKT3 was checked by luciferase reporter gene assay. The results showed that the increase or decrease of miR-320b could significantly suppress or facilitate the luciferase activity in the AKT3-3'-UTR-WT group, but had no effect on AKT3-3'-UTR-MUT group (Fig. 5B, $P < 0.001$). Further, as shown in Fig. 5C, D, the expressions of AKT3 in the serum of patients with hypertension were significantly increased compared with the control group ($P < 0.001$), and the level of AKT3 in Ang II-induced HUVECs was also increased ($P < 0.001$). The expression of AKT3 was inhibited after up-regulation of miR-320b ($P < 0.001$). Pearson analysis proved that there was a significant negative correlation between AKT3 level and miR-320b level in the serum of patients with hypertension (Fig. 5E, $P < 0.001$).

Discussion

More and more evidence shows that the molecular expression of miRNA in the peripheral blood of patients with hypertension has changed significantly, which is closely related to vascular endothelial and myocardial injury caused by hypertension. In this study, we detected that the expression level of miR-320b was differentially expressed in patients with hypertension and healthy people,

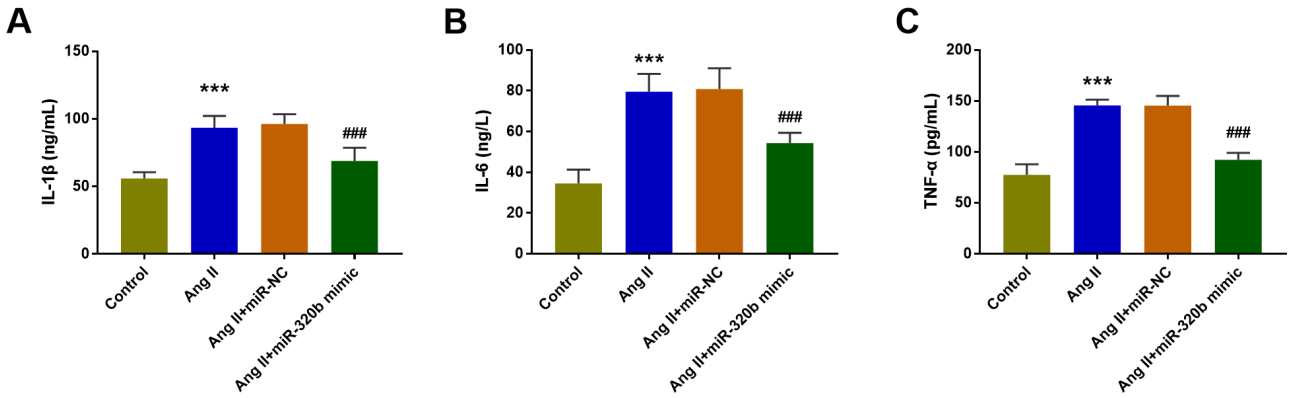

Fig. 4. Effects of miR-320b on the inflammatory response of HUVECs induced by Ang II.
The effects of miR-320b on (A) interleukin-1β (IL-1β), (B) interleukin-6 (IL-6) and (C) tumor necrosis factor-α (TNF-α) in Ang II-induced HUVECs were detected by ELISA (n = 6 per group). $^{***}P < 0.001$ vs. control group; $^{###}P < 0.001$ vs. Ang II group.

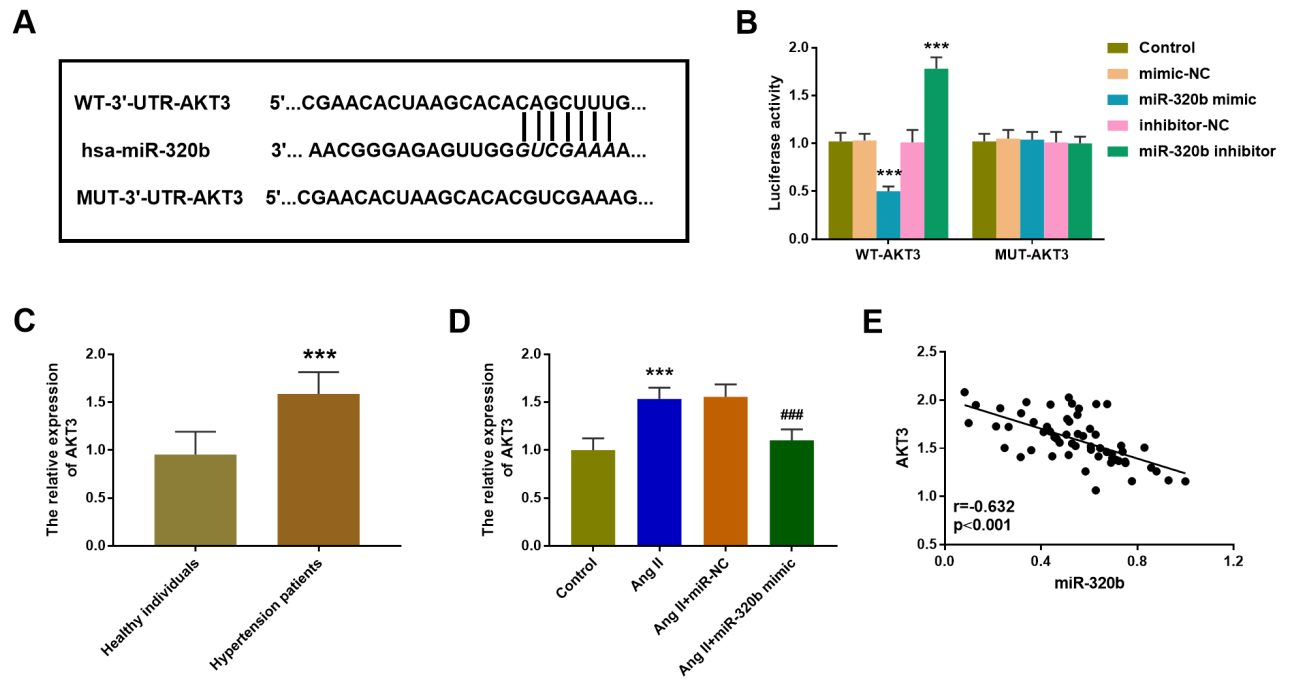

Fig. 5. Validation of targeting relationship between miR-320b and AKT3.
(A) Complementary binding sites of miR-320b and AKT3. (B) Luciferase reporter gene assay. (C) The serum expression levels of AKT3 in all individuals were detected by RT-qPCR. (D) The expression levels of AKT3 in HUVECs were detected by RT-qPCR (n = 3 per group). (E) Correlation between serum AKT3 level and miR-320b level in patients with hypertension was evaluated by Pearson method. $^{***}P < 0.001$ vs. control group; $^{###}P < 0.001$ vs. Ang II group.

and the expression was reduced in patients with hypertension. The reduction of miR-320b was significantly related to the increase of blood pressure and other clinical indicators, and miR-320b also shows its potential value as a diagnostic marker for hypertension. In addition, the overexpression of miR-320b significantly reversed the inhibition of Ang II on HUVECs activity and the promotion of apoptosis and inflammatory response by targeting AKT3 expression *in vitro*.

MiR-320b is a member of the miR-320 family, which is expressed in both humans and primates, but not in rodents (Lu et al. 2022). MiR-320b is a kind of miRNA molecule with multiple biological functions, which is closely related to various tumors, apoptosis, inflammation, and vascular injury. In this study, the expression of miR-320b was decreased in patients with hypertension. There is evidence that miR-320b regulates pathological inflammatory responses by regulating the expression of ICAM-1 in endothelial cells (Gidlof et al. 2013). Xu et al. (2022) confirmed that the expression of hsa-miR-320b was reduced in

chronic thromboembolic pulmonary hypertension. This study is consistent with what we have found so far. In human blood, miRNAs exist in such a stable form that even endogenous ribonuclease cannot affect them. In addition, studies showed that long before the typical clinical symptoms of the disease appear, the miRNAs in the blood may be abnormal. Therefore, the changes in the expression of miRNAs in blood circulation are expected to be valuable biomarkers for the diagnosis of clinical disease. For example, Xu et al. (2021a) reported that miR-637 has predictive value for the cardiovascular events in patients with atherosclerosis, and it is also a potential noninvasive diagnostic biomarker. In this study, ROC analysis revealed that miR-320b had high diagnostic accuracy for hypertension, indicating that this miRNA has the potential to be a candidate diagnostic biomarker for hypertension.

The occurrence of hypertension is an extremely complicated process, which is regulated by multiple factors. At present, it is believed that miRNA participates in the occurrence, development and prognosis of hypertension in different ways. MiR-145 can regulate blood pressure by maintaining the function of vascular smooth muscle cells (Oury et al. 2016). MiR-135a regulates blood pressure by decreasing the gene expression of the mineralocorticoid receptors (Sober et al. 2010; Magenta et al. 2013). Previous studies have mentioned that miR-320b is associated with the changes in the function of vascular endothelial cells. Therefore, this study attempted to construct a vascular endothelial injury model to study hypertension. Many studies have shown that Ang II-induced HUVECs are commonly used in cell models to simulate hypertensive damage (Fu et al. 2021). In this study, the expression of miR-320b and the proliferation of HUVECs were all inhibited while the apoptosis of HUVECs were promoted after Ang II-induction. These results were consistent with the results reported in related studies (Siow 2012), indicating that the cell model of hypertensive damage has been successfully established. In further experiments, it was found that overexpression of miR-320b showed a role in alleviating Ang II-induced endothelial cell damage and inflammatory response.

Mature miRNA needs to combine with the corresponding RNA-induced target gene silencing complex to play its biological role. Therefore, in order to study the mechanism of miR-320b in the occurrence and development of hypertension, we further explored the possible downstream target genes of miR-320b. Further luciferase reporter gene experiments proved the interaction between miR-320b and AKT3. According to the prediction of TargetScan online database, we found that AKT3 may be the target gene of miR-320b. AKT, also known as PKB, consists of the three subtypes: AKT1, AKT2, and AKT3. The dysregulation of AKT is related to cancer, diabetes, cardiovascular disease, and neurological diseases (Hers et al. 2011; Linton et al. 2016). In this study, AKT3 expression was increased in Ang II-induced HUVECs. Studies have shown that a sustained increase in AKT3 can lead to systolic dysfunction of the heart and vascular endothelium injury (Taniyama et al. 2005). In addition, Pearson correlation coefficient analysis showed that the expression level of AKT3 in serum was negatively regulated by miR-320b, which further clarified the relationship between miR-320b and AKT3.

Limitations of this study are as follows: Only 59 patients with hypertension were included in this study, with a small sample size and all from the same hospital, which may not avoid the occurrence of selection bias. Therefore, whether the results of the study are applicable to all patients with hypertension remains to be known, and the sample size needs to be further expanded for relevant verification. In addition, the cell experiment only investigated the role of miR-320b on cell biological functions and did not explore the molecular pathways to explain the mechanism of miR-320b in hypertension. Thus, future studies should focus on exploring the molecular pathway and mechanism of miR-320b regulation of hypertension through further experiments.

In this study, miR-320b was significantly reduced in the serum of patients with hypertension, and this trend showed high accuracy in distinguishing hypertension from healthy individuals. In addition, in *in vitro* studies, overexpression of miR-320b showed a certain ability to alleviate Ang II-induced endothelial cell damage through the regulation of AKT3, which was manifested as reversing the inhibitory effect on cell viability and the promotion effect on apoptosis and inflammation. Generally speaking, the abnormal reduction of serum miR-320b is related to the occurrence of hypertension, which can alleviate the progression of hypertension by regulating the expression of AKT3, and has shown clinical value in the prediction of hypertension.

Conflict of Interest

The authors declare no conflict of interest.

References

Asla, Q., Sarda, H., Lerma, E., Hanzu, F.A., Rodrigo, M.T., Urgell, E., Perez, J.I., Webb, S.M. & Aulinas, A. (2022) 11-Deoxycorticosterone Producing Adrenal Hyperplasia as a Very Unusual Cause of Endocrine Hypertension: Case Report and Systematic Review of the Literature. *Front. Endocrinol. (Lausanne)*, **13**, 846865.

Chen, C., Jin, Y., Lo, I.L., Zhao, H., Sun, B., Zhao, Q., Zheng, J. & Zhang, X.D. (2017) Complexity Change in Cardiovascular Disease. *Int. J. Biol. Sci.*, **13**, 1320-1328.

Choi, W.H., Ahn, J., Um, M.Y., Jung, C.H., Jung, S.E. & Ha, T.Y. (2020) Circulating microRNA expression profiling in young obese Korean women. *Nutr. Res. Pract.*, **14**, 412-422.

Corsten, M.F., Dennert, R., Jochems, S., Kuznetsova, T., Devaux, Y., Hofstra, L., Wagner, D.R., Staessen, J.A., Heymans, S. & Schroen, B. (2010) Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ. Cardiovasc. Genet.*, **3**, 499-506.

Diez, J. (2008) Diagnosis and treatment of myocardial fibrosis in hypertensive heart disease. *Circ. J.*, **72** Suppl A, A8-12.

Esmaillzadeh, A., Zakizadeh, E., Faghihimani, E., Gohari, M. & Jazayeri, S. (2015) The effect of purslane seeds on glycemic

status and lipid profiles of persons with type 2 diabetes: A randomized controlled cross-over clinical trial. *J. Res. Med. Sci.*, **20**, 47-53.

Fan, J., Ren, M., Adhikari, B.K., Wang, H. & He, Y. (2022) The NLRP3 Inflammasome as a Novel Therapeutic Target for Cardiac Fibrosis. *J. Inflamm. Res.*, **15**, 3847-3858.

Fu, Z., Wang, F., Liu, X., Hu, J., Su, J., Lu, X., Lu, A., Cho, J.M., Symons, J.D., Zou, C.J. & Yang, T. (2021) Soluble (pro)renin receptor induces endothelial dysfunction and hypertension in mice with diet-induced obesity via activation of angiotensin II type 1 receptor. *Clin. Sci.* (*Lond.*), **135**, 793-810.

Gao, Y., Qi, G.X., Jia, Z.M. & Sun, Y.X. (2017) Prediction of marker genes associated with hypertension by bioinformatics analyses. *Int. J. Mol. Med.*, **40**, 137-145.

Gidlof, O., van der Brug, M., Ohman, J., Gilje, P., Olde, B., Wahlestedt, C. & Erlinge, D. (2013) Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression. *Blood*, **121**, 3908-3917, S3901-3926.

Hers, I., Vincent, E.E. & Tavare, J.M. (2011) Akt signalling in health and disease. *Cell. Signal.*, **23**, 1515-1527.

Joint Committee for Guideline Revision (2019) 2018 Chinese Guidelines for Prevention and Treatment of Hypertension-A report of the Revision Committee of Chinese Guidelines for Prevention and Treatment of Hypertension. *J. Geriatr. Cardiol.*, **16**, 182-241.

Kriegel, A.J., Baker, M.A., Liu, Y., Liu, P., Cowley, A.W. Jr. & Liang, M. (2015) Endogenous microRNAs in human microvascular endothelial cells regulate mRNAs encoded by hypertension-related genes. *Hypertension*, **66**, 793-799.

Lee, A., McLean, D., Choi, J., Kang, H., Chang, W. & Kim, J. (2014) Therapeutic implications of microRNAs in pulmonary arterial hypertension. *BMB Rep*, **47**, 311-317.

Li, J., Wu, Y. & Liu, H. (2020) Expression and role of miR-338-3p in peripheral blood and placenta of patients with pregnancy-induced hypertension. *Exp. Ther. Med.*, **20**, 418-426.

Linton, M.F., Babaev, V.R., Huang, J., Linton, E.F., Tao, H. & Yancey, P.G. (2016) Macrophage Apoptosis and Efferocytosis in the Pathogenesis of Atherosclerosis. *Circ. J.*, **80**, 2259-2268.

Lu, X., Yang, B., Yang, H., Wang, L., Li, H., Chen, S., Lu, X. & Gu, D. (2022) MicroRNA-320b Modulates Cholesterol Efflux and Atherosclerosis. *J. Atheroscler. Thromb.*, **29**, 200-220.

Magenta, A., Greco, S., Gaetano, C. & Martelli, F. (2013) Oxidative stress and microRNAs in vascular diseases. *Int. J. Mol. Sci.*, **14**, 17319-17346.

Moskalik, A., Ratajska, A., Majchrzak, B., Jankowska-Steifer, E., Bartkowiak, K., Bartkowiak, M. & Niderla-Bielinska, J. (2022) miR-31-5p-Modified RAW 264.7 Macrophages Affect Profibrotic Phenotype of Lymphatic Endothelial Cells In Vitro. *Int. J. Mol. Sci.*, **23**.

Oury, C., Servais, L., Bouznad, N., Hego, A., Nchimi, A. & Lancellotti, P. (2016) MicroRNAs in Valvular Heart Diseases: Potential Role as Markers and Actors of Valvular and Cardiac Remodeling. *Int. J. Mol. Sci.*, **17**.

Siow, R.C. (2012) Culture of human endothelial cells from umbilical veins. *Methods Mol. Biol.*, **806**, 265-274.

Sober, S., Laan, M. & Annilo, T. (2010) MicroRNAs miR-124 and miR-135a are potential regulators of the mineralocorticoid receptor gene (NR3C2) expression. *Biochem. Biophys. Res. Commun.*, **391**, 727-732.

Solayman, M.H., Langaee, T., Patel, A., El-Wakeel, L., El-Hamamsy, M., Badary, O. & Johnson, J.A. (2016) Identification of Suitable Endogenous Normalizers for qRT-PCR Analysis of Plasma microRNA Expression in Essential Hypertension. *Mol. Biotechnol.*, **58**, 179-187.

Taniyama, Y., Ito, M., Sato, K., Kuester, C., Veit, K., Tremp, G., Liao, R., Colucci, W.S., Ivashchenko, Y., Walsh, K. & Shiojima, I. (2005) Akt3 overexpression in the heart results in progression from adaptive to maladaptive hypertrophy. *J. Mol. Cell. Cardiol.*, **38**, 375-385.

Xu, W., Deng, M., Meng, X., Sun, X., Tao, X., Wang, D., Zhang, S., Zhen, Y., Liu, X. & Liu, M. (2022) The alterations in molecular markers and signaling pathways in chronic thromboembolic pulmonary hypertension, a study with transcriptome sequencing and bioinformatic analysis. *Front. Cardiovasc. Med.*, **9**, 961305.

Xu, W., Hong, Q., Zhou, Y., Chen, X., Li, L., Wang, M., Chen, W., Xie, X., Zhuang, D., Lai, M., Zhou, W. & Liu, H. (2023) Circulating plasma and exosome levels of the miR-320 family as a non-invasive biomarker for methamphetamine use disorder. *Front. Psychiatry*, **14**, 1160341.

Xu, Y., Zhao, L., Liu, H., Sun, B. & Zhao, X. (2021a) Diagnostic value of miR-637 in patients with atherosclerosis and its predictive significance for the future cardiovascular events. *Vascular*, **29**, 704-710.

Xu, Z., Ding, J., Zhang, L., Feng, X., Zhou, J., Shen, X., Lu, H., Qian, L. & Li, X. (2021b) Peptidomics analysis revealed that a novel peptide VMP-19 protects against Ang II-induced injury in human umbilical vein endothelial cells. *Mol. Med. Rep.*, **23**.

Yao, H., Pang, Y., Chen, Y., Si, N., Wu, C., Wang, Z. & Ren, Y. (2023) Association Between Interleukin-6 Gene Polymorphism and Severity of Coronary Artery Disease in Patients with Diabetes. *Diabetes Metab. Syndr. Obes.*, **16**, 3599-3608.

Zhang, R., Qin, Y., Zhu, G., Li, Y. & Xue, J. (2016) Low serum miR-320b expression as a novel indicator of carotid atherosclerosis. *J. Clin. Neurosci.*, **33**, 252-258.