rs10719 Polymorphism Located within DROSHA 3’-Untranslated Region is Responsible for Development of Primary Hypertension by Disrupting Binding with microRNA-27b

Yabing Zhang*
Ai-lin Cao*
Chun Dong

* Yabing Zhang and Ai-lin Cao contributed to the study equally

Corresponding Author: Chun Dong. e-mail: hypertensiondrosha@126.com

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Background: MiR-27b is reportedly involved with many diseases (e.g., gastric cancer) by acting on different signaling pathways. In this study, we aimed at understanding the relationship between miR-27b and hypertension and its underlying molecular mechanism.

Material/Methods: Peripheral blood was collected from patients with hypertension, and statistical analysis was performed to study the association between rs10719 and risk of hypertension. Tissue samples were collected from patients with lung cancer, and the expression of miR-27b and DROSHA was determined using Western blot analysis and real-time PCR.

Results: We first searched the miRNA database online, and identified DROSHA as a virtual target of miR-27b with the “seed sequence” located within the 3’-UTR of the target gene, and then validated DROSHA to be the direct gene via luciferase reporter assay system. We also established the negative regulatory relationship between miR-27b and DROSHA via studying the relative luciferase activity. We also conducted real-time PCR to study the mRNA and protein expression level of miR-27b among different groups. Furthermore, we conducted real-time PCR and densitometry analysis to study the mRNA and protein expression level of DROSHA among different groups of cells treated with scramble control, miR-27b mimics, DROSHA siRNA, and miR-27b inhibitors to verify the negative regulatory relationship between MiR-27b and DROSHA.

Conclusions: The presence of rs10719 disrupted the interaction between miR-27b and DROSHA, which might be the underlying mechanism of the observation that rs10719 is significantly associated with risk of primary hypertension.

MeSH Keywords: Cell Proliferation • Hypertension • MicroRNAs • Muscle, Smooth, Vascular

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Background

A variety of diseases, such as vascular stenosis after vessel transplant, atherosclerosis, and hypertension, are contributed to by key compositions, including abnormal proliferation and migration of vascular smooth muscle cell (VSMC) [1]. Furthermore, one of the primary factors that trigger atherosclerotic plaque vulnerability is VSMC apoptosis [2]. The phenotype and function of VSMCs are controlled by hemodynamic factors within the vasculature, including hydrostatic pressure, cyclic strain, and shear stress of the blood vessel wall induced by blood flow [3]. In a normal hemodynamic environment, the apoptosis of VSMC is balanced with proliferation. Unfortunately, the balance between VSMC apoptosis and proliferation is interrupted by aberrant mechanical factors in the blood vessel wall, leading to pathophysiology of the vasculature [4].

As a member of the ribonuclease III superfamily of endoribonucleases, which are double-stranded (ds) and have specific RNA, DROSHA is involved in a variety of RNA maturation and decay pathways in prokaryotic and eukaryotic cells [5]. If DROSHA is missing in VSMCs, the expression of the marker gene of VSMC is reduced in cKO mice [6]. Hence, the expression of VSMC marker gene can be reduced by interruption of the miRNA biogenesis pathways via deletion of DROSHA, and consequently the cell contraction is regulated, which has been demonstrated in Dicer-inducible VSMC-specific knockout and knockout VSMCs mice [6,7]. At present, the molecular mechanism by which DROSHA regulates cell proliferation and differentiation remains unclear.

microRNAs (miRNAs), which consist of about 22 nucleotides, are a class of small non-coding RNA molecules. They bind to the 3′ untranslated region (UTR) of target gene mRNA and hence post-transcriptionally regulate the expression of genes [8–10]. It was originally reported that hsa-miR-27b regulated DROSHA expression in bladder cancer. hsa-miR-27b has been extensively studied, and had been found to be associated with a variety of cancers, including neuroblastoma and colorectal cancer [11]. In a recent study, it was found that hsa-miR-27b suppressed angiogenesis and progression of colorectal tumor via affecting VEGF [11]. In a study by Lee et al., hsa miR-27b was shown to serve as a tumor inhibitor to suppress inflammatory response, tumor progression, and cell growth and by impacting PPARγ 3′UTR present in neuroblastoma cells [12]. hsa-miR-27b is also shown to regulate protein translation, thereby affecting the expression of DROSHA. To determine the true pathway and mechanism in bladder cancer, more functional studies are required [13].

Single-nucleotide polymorphism (SNP) is the variant in the human genome that refers to the substitution of nucleotide, which, when located in the 3′-UTR of a target gene, might interfere with the binding of miRNA and compromises the inhibitory effect of the regulator [14]. The SNP rs10719 has been found to be located in the miR-27b binding site within 3′UTR of DROSHA, and dysregulation of DROSHA has been shown to be associated with abnormal proliferation of smooth muscle cells [13]. Based on the above-mentioned evidence, we hypothesized that rs10719 may interfere with the interaction between DROSHA mRNA and miR-27b, and the minor allele of rs10719 might be associated with risk of primary hypertension. In this study, we collected tissue samples and peripheral blood sample to test this hypothesis.

Material and Methods

Tissue sample collection

In this study, 5 patients with diagnosed lung cancer who received surgical treatment were recruited from Feb in 2014 to Nov in 2014 in the First People’s Hospital of Jining (Jining, China). Adjacent (at least >2 cm) non-cancerous tissue samples were collected and stored in liquid nitrogen for future use.

Peripheral blood collection

Peripheral blood samples from 623 patients with hypertension were collected between June 2013 to Nov 2014 in the First People's Hospital of Jining (Jining, China). The demographic and clinicopathological characteristics of the participants are described in Table 1. The study protocol was approved by the Research Ethics Committee at the First People’s Hospital of Jining (Jining, China), and all participants provided written consent prior to study initiation.

DNA isolation and genotyping by direct sequencing

We mixed 100 μL of binding buffer (High-Cutoff, Invitrogen, Carlsbad, CA) with the tissues or whole blood in PBS. Proteins and all other cell lysis solution were removed through the pores by using 100 ul washing buffer (Invitrogen), while DNA remained on the surface of silica. Finally, 5–20 μL of elution buffer (10 mM Tris-HCl, pH 8.5) was used to obtain target DNA (DROSHA), which was collected into a microcentrifuge tube.

Rs10719 was evaluated for all of the tissue samples. The BigDye3 terminator cycle sequencing kit (PE Applied Biosystems) was used to sequence the chromosome segment containing rs10719, in accordance with manufacturer’s instructions. We used an ABI PRISM 3100 Genomic Analyser (PE Applied Biosystems) to perform the sequencing.

RNA isolation and real-time PCR

Trizol (Invitrogen, Carlsbad, CA) was used to extract total RNA from PASMCS or tissue in accordance with the manufacturer’s
protocol. For miRNA analysis, the Hairpin-it™ miRNAs RT-PCR Quantitation Kit (GenePharma, Shanghai, China) was used to analyze the real-time PCR. \( -\Delta \Delta Ct \) method was used to analyze the relationship between the expression of miR-27b and the U6 small nuclear RNA. To analyze the expression of mRNA, the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Madison, WI) was used to perform complementary cDNA synthesis using SYBR PremixExTaq (Takara, Madison, WI) under standard conditions. We conducted real-time PCR in accordance with the manufacturer’s protocol. The CFX96Real-Time PCR Detection System (BioRad, USA) was used to detect the result of the real-time PCR, and analyzed using the \( -\Delta \Delta Ct \) method was used to analyze the result with GAPDH as the internal control. A Mini Opticon Real-time PCR System (BioRad) was used to perform the real-time PCR. The 10-ml PCR mixture contained 0.7 mM reverse primer, 0.2 mM TaqMan probe, 15 mM forward primer 1.33 mL RT product, and 2×TaqMan Universal PCR Master Mix (no AmpErase UNG). All reactions were maintained in a 48-well plate at 95°C for 10 min, and then 40 cycles at 95°C for 15 s and 60°C for 60 s and repeated 3 times. Applied Biosystems 7500 sequence detection software was used to analyze the expression of miR-27b.

Cell culture and transfection

PASMCs were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 mg/ml Streptomycin, and 100 u/ml penicillin (GIBCO; Invitrogen) in an atmosphere of 5% CO\(_2\) in air at 37°C. PASMCs were transferred into 6-well plates contained growing medium without antibiotic for 12 h at a density of 3–6×10\(^5\) cells/well before transfection. When confluence reached 30–50%, we transfected the PASMCs with Lipofectamine™ 2000 transfection reagent (Invitrogen, USA) in accordance with the manufacturer’s protocol. The experiments were performed in triplicate.

Vector construction and mutagenesis

Sequences containing miR-27b target site in 3’ end of DROSHA gene were amplified by PCR with specific primers for cloning into pMIR-REPORT vector (Ambion) at HindIII and SpeI restriction sites. miRNA seed sequence mutation was performed using the QuikChange® Lightning Site-Directed Mutagenesis kit (Agilent Technologies) with carefully designed primers according to the instructions, and direct sequencing was used to confirm the mutation site accuracy.

Cell proliferation assay

CCK8 assay was used to detect the viability of cell CCK8 assay, in accordance with the manufacturer’s protocol. In brief, the cells were transferred into 96-well plates at a density of 4000 cells per well for 12 h after transfection. Then we added 10 μL of CCK8 into each well, and maintained the culture medium at 37°C for another 3 h. ELISA was used to analyze the absorbance at 450 nm wavelength. Mitochondrial activity indirectly reflects living cell numbers and cell proliferation according to the absorbance at 450 nm. All experiments were performed at least 3 times.

### Table 1. Comparison of general information in both groups.

| Items                      | Control group (N=621) | Case group (N=623) | P value |
|----------------------------|-----------------------|--------------------|---------|
| Sex (Male/Female)          | 420/201               | 461/162            | 0.656   |
| Age                       | 64.2±13.3             | 63.8±13.5          | 0.827   |
| TC (mM)                   | 4.02±1.27             | 4.12±1.15          | 0.663   |
| TG (mM)                   | 1.26±0.36             | 1.29±0.44          | 0.712   |
| HDL-C (mM)                | 1.72±0.65             | 1.98±0.63          | 0.593   |
| LDL-C (mM)                | 2.78±0.56             | 2.67±0.51          | 0.81    |
| Fasting blood sugar (mM)  | 5.23±0.73             | 5.22±0.57          | 0.542   |
| SBP                       | 127.8±14.8            | 173.7±16.3         | 0.0021  |
| DBP                       | 75.6±10.8             | 98.6±11.7          | 0.0023  |
| Rs10719 genotype          |                       |                    |         |
| TT                        | 328 (52.8)            | 279 (44.9)         | 0.0046  |
| TC                        | 240 (38.6)            | 278 (44.7)         |         |
| CC                        | 53 (8.6)              | 66 (10.4)          |         |
| TC/CC                     | 293 (47.2)            | 344 (55.1)         |         |

Compared with the control group, P<0.05.
Luciferase assay (3'UTR miRNA)

RPMI1640 medium with 10% fetal bovine serum was used to incubate PASMCS cells. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used to transfected miR-27b into PASMCS cells in 24-well plates at a final concentration of 100 nM per well, about 0.4 mg of firefly luciferase reporter vector including the mutant target site, and wild-type and 0.02 mg of the control plasmid including Renilla luciferase pRL-CMV (Promega). The Dual Luciferase Reporter Assay System (Promega) was used to analyze the luciferase assays 12 h after transfection. All experiments were performed at least 3 times.

Western blot analysis

Lysis buffer (Biosharp, Hefei, China) including 5 g/L sodium deoxycholate, 0.2 g/L NaN3,10 mL/L NP-40, 150 mmol/L NaCl 100 μg/mL, 0.1 g/LSDS, phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 50 mmol/L Tris-HCl (pH 8.5) was used to lyse the cells or tissues according to the manufacturer’s instructions. We used 12% SDS-PAGE to purify protein samples and then transferred them to nitrocellulose membranes (GE Healthcare, Milan, Italy). To avoid unspecified binding, Tris-buffered saline/Tween-20 (0.1%, Bioeasy, Shanghai, China) including 5% non-fat milk (Merck, Darmstadt, Germany) was used to incubate with the membrane at room temperature for 2 h. Subsequently, monoclonal antibodies against DROSHA (anti-DROSHA antibody, 1:1000, RT, 2 h, Abcam, Boston, MA) were incubated with the blot, and at the same time a monoclonal antibody against β-actin (anti-β-actin antibody, 1:10000, RT, 1 h, Abcam, Boston, MA) were used as an internal control. After washing with PBS (Invitrogen, CA), secondary antibody conjugated to HRP (1:10000, RT, 1 h, Abcam, Boston, MA) and the ECL Western Blotting Kit (Promega, Milan, Italy) were used for signal detection according to the manufacturers’ protocols.

Apoptosis analysis (flow cytometry)

Flow cytometry was used to detect and quantify the apoptotic rate of the PASMCS cells, by staining with Annexin V-FITC (Roche, Mannheim, Germany) and PI (propidium iodide) (Roche, Mannheim, Germany). After harvesting, the PASMCS cells (1×10⁶) were washed with PBS (Greiner, Balhingen, Germany) and incubated with Annexin V-PI (Roche, Mannheim, Germany) at room temperature for 15 min in the dark. A Becton Dickinson fluorescence-activated cell sorter (BD Biosciences, San Jose, CA) with 585/42 nm (PI) and 530/30 nm (FITC) emission filters was used to detect the PASMCS cells. Becton Dickinson Cell Quest software was used to analyze the percentage of apoptosis. Three independent experiments were performed.

Statistical analysis

SPSS 13.0 statistical software (SPSS Inc, Chicago, IL) was used to perform statistical analysis. The t test was used for comparisons between 2 groups, and the one-way ANOVA and Student-Newman-Keuls test were performed for multiple comparisons. Chi-square test and logistic regression analysis were used to determine the association between the polymorphism and risk of primary hypertension. P value <0.05 was considered to indicate statistical significance. All results are shown as means ±SD (standard deviation).

Results

DROSHA was virtual target of miR-27b

It has been previously shown that DROSHA is a validated target of miR-27b in other human cell types. Based on the computational analysis, the DROSHA polymorphism was found to be located within predicted binding sites for hsa-miR-27b (Figure 1). To test whether hsa-miR-27b targets DROSHA 3'UTR in SMC cells, we constructed reporter vectors carrying wild-type or mutant-1 and mutant-2 DROSHA 3'UTR, as described in Figure 2. Subsequently, we used them for transient transfection with miR-27b mimics, together with wild-type DROSHA, mutant-1, and mutant-2 DROSHA or scramble controls. As shown in Figure 2, only the luciferase activity from the SMC cells cotransfected with wild-type DROSHA 3'UTR and miR-27b mimics was significantly lower than the control, and all other groups were comparable. Our data indicate that DROSHA is a validated target of miR-27b in SMC cells.

Determination of expression patterns of miR-27b and DROSHA in lung tissue with different genotypes

We then collected lung tissues from the patients with lung cancer who received surgical intervention. The tissues were genotyped for rs10719 (CC, n=32, CT, n=18, TT, n=5) and used to further explore the impacts of the polymorphism on the interaction between miR-27b and DROSHA 3'UTR. Using real-time PCR, we found the expressions of miR-27b were comparable among all groups (Figure 3). We then detected DROSHA mRNA and protein expression using real-time PCR and densitometry analysis. We found DROSHA mRNA (Figure 4A) and protein (Figure 4B) expression decreased in CC groups compared with CT and TT groups. To further understand the regulatory relationship between miR-27b and DROSHA, we detected the expression level of mRNA of miR-27b and DROSHA in all the tissues (n=55), then we conducted correlation analysis and found a negative regulatory relationship between miR-27b and DROSHA (r=-0.4218, P<0.05) (Figure 5).
Effect of miR-27b mimics/inhibitors on the expression of DROSHA

To further validate the hypothesis of the negative regulatory relationship between miR-27b and DROSHA, we investigated the mRNA/protein expression level of DROSHA of SMC cells by transfecting the SMC cells with scramble control, miR-27b mimics, DROSHA siRNA, and miR-27b inhibitors. As shown in Figure 6, the DROSHA protein (Figure 6A) and mRNA expression level (Figure 6B) of SMC cells treated with miR-27b mimics and

Figure 2. The luciferase activity from the SMC cells cotransfected with wild-type DROSHA 3’UTR and miR-27b mimics was significantly lower than in the controls, and all other groups were comparable.

Figure 3. The expression of miR-27b was comparable among the 3 different genotypes (CC, n=32, CT, n=18, TT, n=5).

Figure 4. The expression of DROSHA mRNA (A) and protein (B) decreased in CC groups compared with CT and TT groups.

Effect of miR-27b mimics/inhibitors on the expression of DROSHA

To further validate the hypothesis of the negative regulatory relationship between miR-27b and DROSHA, we investigated the mRNA/protein expression level of DROSHA of SMC cells by transfecting the SMC cells with scramble control, miR-27b mimics, DROSHA siRNA, and miR-27b inhibitors. As shown in Figure 6, the DROSHA protein (Figure 6A) and mRNA expression level (Figure 6B) of SMC cells treated with miR-27b mimics and
DROSHA siRNA were clearly lower than in the scramble control, while cells treated miR-27b inhibitors were noticeably higher than in the scramble control. The results validated the negative regulatory relationship between miR-27b and DROSHA.

**Association study between rs10719 and development of primary hypertension**

To investigate the association between the rs10719 and PD, we collected whole peripheral blood from individuals, including individuals with primary hypertension (n=623) and normal controls (n=621), and genotyped for rs10719. As shown in Table 1, we found that rs10719 is significantly associated with the risk of primary hypertension in a Chinese population using logistic regression analysis (OR=1.38, 95% CI [1.10–1.72], P=0.004).

**Discussion**

In this study, we identified DROSHA as a target of miR-27b and validated DROSHA to be the direct gene via luciferase reporter assay system. We collected whole peripheral blood from individuals with primary hypertension (n=623) and from normal controls (n=621) and genotyped for rs10719, finding that rs10719 is significantly associated with the risk of primary hypertension in a Chinese population.

Several teams have discovered the role that miR-27b plays in angiogenesis regulation [15]. Its targets related with angiogenesis have been identified, including Sprouty-2, Sema6A, and Dll4 [15]. A review indicates that VEGF signaling and sprouting angiogenesis in vitro and in vivo is reduced by silencing of miR-27b. Several studies have suggested that it is associated with cardiovascular development, where it leads to cardiac hypertrophy and left ventricular maturation, and is necessary for early venous specification of the vascular endothelium [15–17]. It plays a role in the homeostasis of vascularity, which is evidenced by its regulation by pulsatile shear stress. Consistently, reduction of neonatal retinal angiogenesis and choroidal neovascularization induced by laser were observed when miR-23 and miR-27b in the miR-23/27/24 cluster were reduced and were simultaneously knocked-down [17]. In response to hyperlipidemia, miR-27b in the liver is augmented and, consequently, modulates pivotal genes that control the metabolism of lipid (Angptl3, Gpam) [18]. Furthermore, in mouse models of dyslipidemia/atherosclerosis, the expression of miR-27b in the liver is reduced and the expressions of its targets are increased [18]. Also, the reduction of miR-27b is observed in differentiating adipocytes, which inhibits their proliferation and build-up through pathways controlled by PPARY/RXRα [19]. In this study, we searched the miRNA database online (www.mirdb.org) and identified DROSHA as
a virtual target of miR-27b with the “seed sequence” located within the 3'-UTR of the target gene, and then validated DROSHA to be the direct gene via luciferase reporter assay system. We also determined the expression level of miR-27b and DROSHA mRNA, and established the negative regulatory relationship between miR-27b and DROSHA by using real-time PCR, which further confirmed the regulatory association between miR-27b and DROSHA.

DROSHA is known as a member of the RNase III enzyme family and is a primary regulator of miRNAs. It is the pivotal nuclease that processes pri-miRNAs into pre-miRNAs in the nucleus as part of the miRNA biogenesis pathway [20]. The generation of miRNAs involves a 2-step processing pathway mediated by 2 master enzymes (DROSHA and DICER). In the nucleus, RNase II processes the longer precursors into primary RNAs (pri-miRNAs). Subsequently, RNase enzyme (DROSHA) processes the pri-miRNAs into precursors (pre-miRNAs) which have a stem-loop structure [21]. The exportin-5 protein transports pre-miRNAs from the nucleus to the cytoplasm. In the cytoplasm, another RNase enzyme (DICER) processes pre-miRNAs into mature miRNAs. The mature miRNAs incorporate into the RNA-induced silencing complex (RISC) and thereby exhibit their effects [22]. It is estimated that 30% of human genes are regulated by miRNAs [23]. miRNAs have been shown in recent several studies to function as tumor suppressors and oncogenes by impacting the target known as 3'UTR of important genes, and miRNA-mediated gene regulation is affected by the genetic variants in 3'UTR of the miRNA, finally leading to higher risk of cancer [24]. Notably, DROSHA and DICER are very important in carcinogenesis. Increasing evidence demonstrates that imbalanced expression levels of DROSHA and DICER are correlated with the risk for bladder cancer [25]. A recent study of Han et al. showed that the bladder cancer tissues had higher expression level of DROSHA and DICER than the control (normal) bladder tissues, and silencing DROSHA or DICER can trigger cell apoptosis and suppress cell proliferation [26].

It has been suggested that a few of 3'UTR polymorphisms may be present in the vicinity of the binding site or the miRNA binding site and probably interrupt function of miRNA, resulting in different gene expressions affecting the occurrence of cancer [24]. It has been demonstrated that substitution of DROSHA rs10719 T by C destroyed a binding site for hsa-miR-27b, leading to elevated expression of DROSHA 3'UTR luciferase [27]. Therefore, there is a correlation between the C allele and higher risk of bladder cancer, likely contributing to elevated expression of DROSHA, which was in consistency with the previous result [26]. A correlation of rs7737174 AA genotype/DROSHA rs644236 TT genotype with the risk of breast cancer in postmenopausal women has been reported [28]. According to the One Thousand Genomes data, DROSHA 3'UTR rs10719 is in strong linkage disequilibrium with rs644236. In the present study we collected lung tissues from the patients with lung cancer who received surgical intervention. The tissues were genotyped for rs10719 (CC, n=32, CT, n=18, TT, n=5) and used to further explore effects of the polymorphism on the interaction between miR-27b and DROSHA 3'UTR. Using real-time PCR, we found the expression of miR-27b was comparable among all genotype groups (Figure 3). We then detected DROSHA mRNA and protein expression by using real-time PCR and densitometry analysis. We found that DROSHA mRNA (Figure 4A) and protein (Figure 4B) expression decreased in CC groups compared with CT and TT groups. Furthermore, we collected whole peripheral blood from individuals, including those with primary hypertension (n=623) and normal controls (n=621), and genotyped for rs10719. Using logistic regression analysis, we found that rs10719 is significantly associated with the risk of primary hypertension in a Chinese population (Table 1) (OR=1.38, 95% CI [1.10–1.72], P=0.004).

Conclusions

The findings show that DROSHA is a virtual target of miR-27b, there is a negative regulatory relationship between miR-27b and DROSHA, and the presence of rs10719 disrupted the interaction between miR-27b and DROSHA, which might be the underlying mechanism of the observation that rs10719 is significantly associated with increased risk of primary hypertension.

Conflict of interest

The authors declare no conflict of interest.

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