Dysregulation of miR-637 Is Involved in the Development of Retinopathy in Hypertension Patients and Serves a Regulatory Role in Retinol Endothelial Cell Proliferation

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Keywords

MiR-637 · Hypertensive retinopathy · Diagnosis · Retinal endothelial cell · STAT3

Abstract

Background: MicroRNAs play an important role in the proliferation and migration of retinal endothelial cells in patients with hypertension and hypertensive retinopathy (HR). This study aimed to investigate the clinical value of miR-637 in HR and its role in retinal endothelial cell proliferation and migration. **Methods:** A total of 126 subjects were recruited for the study, including 42 patients with hypertension (male/female 25/17), 42 healthy individuals (male/female 20/22), and 42 cases with HR (male/female 20/22). Except SBP and DBP, there was no significant difference in other indexes among the three groups. Quantitative real-time PCR was used to detect the expression of miR-637. The receiver operating curve (ROC) was used for diagnosis value analysis. Logistic regression analysis was used to evaluate the relationship between miR-637 and HR. CCK-8 and Transwell were used to detect the effect of miR-637 on the proliferation and migration of human umbilical vein endothelial cells. **Results:** Compared with hypertensive patients, HR patients had the

lowest expression of miR-637. The area under the curve of miR-637 detected by the ROC curve method is 0.892, which has the ability to distinguish hypertension and HR patients. Logistic regression analysis showed that miR-637 was an independent influencing factor in HR. Cell experiment results showed that overexpression of miR-637 significantly inhibited cell proliferation and migration, while downregulation of miR-637 had the opposite effect. Luciferase analysis showed that STAT3 was the target gene of miR-637. **Conclusion:** Our data indicate that miR-637 is a potential noninvasive marker for patients with HR. The action of miR-637 on STAT3 may inhibit the proliferation and migration of retinal endothelial cells, providing a possible target for the treatment of HR.

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Introduction

Hypertension is a main independent risk factor in cardiovascular disease and mortality worldwide [1]. One of the complications of hypertension is retinopathy, which

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can not only reflect the pathological degree of systemic blood vessels and organs such as the heart, brain, and kidney but also is one of the main causes of vision loss and even blindness [2]. Endothelial cells form a thin inner layer that serves as an anatomical and functional interface between the circulating fluid in the cavity and the rest of the vessel wall. The main functions of endothelial cells include regulation of vascular tone, fluid filtration, cell recruitment, hormone transport, and hemostasis [3]. It is worth noting that, according to reports, endothelial function in patients with hypertension is impaired [4]. In addition, microvascular changes in hypertensive retinopathy (HR) are also indicators of other end-organ damage and clinical events [5].

The mass data have proven that microRNAs (miRNAs) can regulate gene expression via translation inhibition and post-transcriptional degradation [6]. The relative role of different miRNAs in vascular biology is a potential regulator of post-transcription. Therefore, miRNAs may become potential biomarkers or drug targets in vascular remodeling [7]. For example, Santulli et al. demonstrated in miRNAs and endothelial cell (DyS) function studies that miR-637 expression analysis can distinguish the white coat and non-white coat hypertension individuals [7]. A study about the upregulation of circular RNA has-circ-0037909 promotes essential hypertension mentioned that since endothelial cells are related to the pathology of target tissue damage from hypertension, Bao et al. [8] used TNF- α to induce the formation of EH mimics and examined changes about has-circ-0037909 and hsa-miR-637. Hence, a comprehensive understanding of the role of miRNA in endothelial dysfunction and hypertension and the molecular mechanism of miRNA action may provide novel diagnostic biomarkers and therapeutic targets for controlling hypertension-related endothelial dysfunction and other cardiovascular complications. According to the research progress, this paper mainly studies the expression level of miR-637 in patients with HR and discusses its clinical application value and its effect on retinal endothelial cell proliferation and migration.

Materials and Methods

Subjects

This study was approved by the Research Ethics Committee of Weifang People's Hospital (approval no. 2018437). Subjects were informed of the research purpose and experimental procedures before participating and signed the consent form approved by the Ethics Committee. The research method complies with the standards stipulated in the Declaration of Helsinki.

126 individuals were recruited, of which 42 cases were in the healthy group (52.24 ± 3.89 years old, male/female 20/22), 42 cases were in the hypertension group (52.26 ± 4.94 years old, male/female 25/17), and 42 were in the HR group (52.95 ± 4.71 years old, male/female 20/22). The diagnosis of hypertension was determined in accordance with the definition of the World Health Organization, according to which hypertension is defined as the systolic blood pressure level at >140 mm Hg and/or diastolic blood pressure at >90 mm Hg [9]. Standard mercury sphygmomanometer was used to measure both systolic and diastolic blood pressure in the left arm. Blood pressure was recorded three times, and their mean values were taken. All hypertension patients were diagnosed for more than 5 years and received hypertension-related medication (such as irbesartan, carvedilol, nifedipine). Patients who had a history of coronary heart disease, smoking, diabetes, cardiovascular and cerebrovascular disease, and cancer were excluded.

Clinical Samples Collection

All participants underwent ophthalmology examinations according to the Eighth Report of the Joint National Committee. Blood samples were collected between 8:30 and 9 o'clock, and after one night of fasting, to avoid differences in day-night changes. Enzyme colorimetry (Roche Diagnostics, Mannheim, Germany) was used to determine lipid profiles. Fasting blood glucose levels were measured by enzyme colorimetry (GLU, Roche Diagnostic GmbH).

Total RNA Extraction and Quantitative Real-Time PCR Assay

Total RNA was isolated from different cells and serum using TRIzol reagent according to the instructions. The isolated RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix kit (Cat# RR036A, Takara, Dalian, China). In addition, quantitative real-time PCR was performed on an ABI7300 (Applied Biosystems, Foster City, CA, USA) thermal cycler using SYBR mixture and ROX detection kit (CW2602M; CW Biotech, Beijing, China). Using a comparative CT ($2^{-\Delta\Delta C_t}$) method to detect the relative miR-637 expression, the relative gene expression of U6 was used as the internal control.

Cell Culture and Transfection

Human umbilical vein endothelial cells (HUVECs) were purchased from Bena Culture collection (Beijing, China), cultured in DMEM medium containing 10% FBS and cultured in a 37°C, 5% CO₂ constant temperature incubator. In order to regulate the expression level of miR-637, cells were transfected with miR-637 mimic, miR-637 inhibitor, or their negative control (mimic NC, inhibitor NC), which was produced by Ribo Bio (Guangzhou, China). Lipofectamine 2000 (DIR, 11668-500; Invitrogen, Carlsbad, CA, USA) was transfected according to the manufacturer's instructions.

Cell Proliferation Assay

CCK-8 method was used to detect cell proliferation. HUVECs in the logarithmic growth phase were seeded in each well of a 96 plate at 5×10^3 . Cells were detected at 0 h, 12 h, 24 h, and 48 h. Before testing, 10 μ L CCK-8 reagent (DIR, abx150974; Shanghai Beyo time, China) was added and incubated for 2 h in an incubator. The optical density was measured at 490 nm using an iMark microplate absorbance meter (ELx800; Bio-Rad Laboratories, Hercules, CA, USA).

Table 1. Demographic, clinical, and laboratory characteristics among groups

Parameters	Healthy	Hypertension	HR	<i>p</i> value
<i>n</i> (male and female)	42 (20/22)	42 (25/17)	42 (20/22)	0.415
Age, years	52.24±3.89	52.26±4.94	52.95±4.71	0.715
BMI, kg m ⁻²	24.93±1.65	25.37±1.80	24.88±1.64	0.331
SBP, mm Hg	110.19±12.97	148.97±5.80	154.93±5.73	<0.001
DBP, mm Hg	75.53±8.42	100.83±5.78	110.38±8.77	<0.001
Fasting glucose	87.15±5.13	86.78±5.89	87.25±6.03	0.924
Total cholesterol	169.43±12.38	166.60±11.48	175.42±12.61	0.095
LDL, mg dL ⁻¹	84.50±6.17	85.21±5.87	84.33±6.81	0.792
HDL, mg dL ⁻¹	49.07±5.25	48.88±5.59	48.81±4.82	0.972
Triglyceride, mg dL ⁻¹	128.07±10.06	129.55±9.56	125.31±9.90	0.146

Data are expressed as *n* or mean±SD. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HR, retinopathy in hypertensive patients.

Fig. 1. Expression level of miR-637 in different groups was detected by qRT-PCR. Compared with the healthy group and hypertension patients, the expression level of miR-637 in HR patients was significantly decreased. ****p* < 0.001, compared with the healthy control group; ###*p* < 0.001, compared with hypertension group. qRT-PCR, quantitative real-time PCR.

Cell Migration Assay

The cell migration was measured using a Transwell cell (Corning Corporation) with a pore size of 8 μm. Stably transfected cells (5×10^4 cells/well) were seeded in the upper chamber of a serum-free DMEM Petri dish, and the lower chamber was filled with DMEM Petri dishes containing 10% fetal bovine serum as an attractant. At 37°C, post-incubation cells were soaked in 0.1% crystal violet at room temperature for 20 min after 24 h. Then an inverted microscope was used to observe the stained cells (Olympus Corporation Tokyo, Japan) and calculate the view from each field in the random five. The average number of migrated or invaded cells was calculated.

Luciferase Reporter Assay

The candidate target gene of miR-637 was predicted by using Target Scan Release 7.0 (<http://targetscan.org/>) and was

Fig. 2. ROC curve was established to assess the discriminative ability of miR-637 for identifying hypertensive patients and HR patients. The AUC was 0.892, with a sensitivity of 83.33% and specificity of 83.33%, at the cut-off value of 0.630. AUC, area under the curve.

verified by the luciferase reporter assay. The wild type (WT) or mutant type of miR-637 in the 3'-UTR of STAT3 and miR-637 mimic or miR-637 inhibitor were co-transfected into HUVEC cells in each group. Lipofectamine 2000 (Invitrogen, USA) was used for cell transfection. The relative luciferase activity was measured by Dual-Luciferase Reporter System (Promega Corporation, USA) according to the instructions of the manufacturer. Renilla fluorescence activity was identified as the internal reference.

Fig. 3. Effect of miR-637 on the proliferation and migration of retinal endothelial cells. **a** Transfection of miR-637 mimics significantly increased the expression of miR-637, while miR-637 expression was significantly down-regulated after miR-637 inhibitor transfection. **b** Overexpression of miR-637 significantly inhibited cell proliferation, but downregulation of miR-637 had the opposite effect. **c** The number of migrated cells decreased significantly after overexpressing miR-637, and the number of migrated cells increased significantly after the downregulation of miR-637. *** $p < 0.001$, ** $p < 0.01$.

Table 2. Association of different variables with the occurrence of HR

Variables	OR	95% CI	p value
MiR-637	0.290	0.103–0.815	0.019
Gender	0.531	0.190–1.484	0.227
Age	0.718	0.255–2.020	0.530
BMI	1.432	0.529–3.877	0.480
SBP	3.006	1.061–8.517	0.038
DBP	1.941	0.645–5.843	0.238
Fasting glucose	0.947	0.319–2.810	0.922
Total cholesterol	0.678	0.242–1.903	0.461
LDL	1.164	0.420–3.226	0.770
HDL	2.578	0.847–7.604	0.086
Triglyceride	2.162	0.736–6.350	0.161
History of anti-HT	0.632	0.222–1.798	0.390

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HR, hypertensive retinopathy; HT, hypertension.

Statistical Analysis

All data in this study were statistically analyzed using GraphPad Prism 6.0 software and SPSS21.0 software, expressed as mean and standard deviation (SD). Student's *t* test was used to compare the statistical differences of continuous variables between the two groups, and the χ^2 test was used to examine gender differences between the two groups. One-way ANOVA analysis was used to compare the statistical differences among different groups. Logistic regression analysis was used to assess the relationship between different variables and the occurrence of HR. The receiver operating curve (ROC) was used to evaluate the diagnostic value of miR-637. Pearson correlation analysis was used to detect the correlation between miRNA and clinical indicators. $p < 0.05$ was considered statistically significant.

Results

Clinical Characteristics of Different Patient Groups

Table 1 reports the main characteristics of the study population and laboratory results. There was no difference in age, gender distribution, and BMI among the groups. Similarly, due to the results of the selection process, the metabolic parameters between the study groups were also the same. However, SBP and DBP had significant differences between the groups.

Expression Levels of miR-637 in Different Patient Groups

We first studied the serum levels of miR-637 in healthy controls, hypertension groups, and HR groups. The results of the study were shown in Figure 1. The expression level of miR-637 in the serum of patients with hypertension was significantly lower than that of the healthy control group ($p < 0.05$). In addition, this study also found that the expression level of miR-637 in patients with HR was significantly lower than that in patients with hypertension ($p < 0.05$). It was worth noting that compared with the other two groups, the expression level of HR patients was the lowest. The results indicated that miR-637 may be a key biomolecule for HR and play an important biological role in its disease progression.

Diagnostic Value of miR-637 in Patients with HR

According to the expression level of miR-637 in HR patients and a hypertension group, the ROC curve was drawn to evaluate the diagnostic value of miR-637 in HR patients.

According to the TargetScan analysis results, the binding site of miR-637 in STAT3 was shown in Figure 4a. Luciferase reporter assay results showed that miR-637 mimic significantly inhibited luciferase activity of WT 3'-UTR of STAT3 ($p < 0.01$), and miR-637 inhibitor significantly increased its luciferase activity (shown in Fig. 4b). In addition, the luciferin activity of the mutant type group was not affected by the transfection of miR-637 mimic or miR-637 inhibitor (shown in Fig. 4b).

Discussion

As previous studies described, many diseases can cause changes in retinal signals, such as hypertension, diabetes, metabolic syndrome, and heart disease [10]. HR is an important complication and is the main site of target organ damage caused by hypertension. At the same time, knowledge of HR is also important in the cardiovascular risk stratification of hypertensive individuals [11]. As we all know, when the blood pressure exceeds the critical limit, the automatic regulation of retinal circulation will fail [12]. Thus, determining clinical characteristics that can be used to predict the development and progression of retinopathy is essential for hypertensive patients. For example, a study mentioned that retinal vascular tortuosity and arterial narrowing due to HR and arteriolar sclerosis were noted in both eyes [13]. The specific miRNA can destroy the blood vessel-retinal barrier and selectively secrete it into the circulatory system through small membrane bubbles (such as exosomes) [14]. Our results showed that the expression level of miR-637 was highest in the healthy group without hypertension and lowest in the HR patient. Yin et al. [15] confirmed that miR-637 expression is reduced in hypertensive subjects. This is consistent with the results reported in the literature. In the meantime, we speculate that miR-637 may have a potential role in the development of the HR population.

Previous studies also confirmed that specific miRNA expression patterns and levels can reveal physiological and pathological changes and related microvascular and macrovascular complications in patients with HR and are considered to be a valuable novel biomarker [16]. For example, in a study about the role of miRNA in endothelial dysfunction and hypertension, it was reported that miR-637 targets other inflammatory pathways in order to decrease the C-reactive protein (CRP) levels in other pathological situations such as hypertension [17]. In another study, the downregulation

of the miR-637, which regulates the CDK6 expression from lung smooth muscle cells, increases the risk of hypoxia-induced pulmonary hypertension [18]. In our study, the ROC curve was used to assess whether serum miR-637 was capable of identifying HR patients from hypertensive cases. The results showed that miR-637 had a high diagnostic capacity between the two groups. Logistic regression analysis showed that miR-637 was an independent influencing factor for the occurrence of HR in hypertensive patients. Therefore, it may be used as a biomarker for HR patients. In addition, studies have confirmed that hypertension can cause changes in miRNAs such as miR-21, miR-122, and miR-637 [19]. This is consistent with the results of this study.

Endothelial cell dysfunction is the main feature of retinal neovascular disease, which is the main cause of blindness in developed countries [20]. Considering the crucial role of endothelial cell dysfunction in the development of HR, the dysregulation of miR-637 in HR patients, we assumed that miR-637 may regulate the proliferation and migration of retinal endothelial cells during the occurrence of HR. Cell experiments confirmed the effect of miR-637 on the proliferation and migration of HUVEC cells, suggesting that miR-637 may be involved in HR through regulating the proliferation and migration of HUVEC cells.

Signal transducer and activator of transcription protein-3 (STAT3) is a transcription factor involved in many biological processes, especially the process of cell survival and proliferation [21]. It was originally isolated from cytokine-induced cell culture, and it was later found in many normal and pathological tissues [22]. For example, it was mentioned in a study that the oxygen-induced retinopathy rat model showed increased neovascular retinal blood vessels with increased STAT3 activity [23]. Last but not least, studies have confirmed that the proliferation of endothelial cells induced by VEGF can be inhibited by STAT3 knockdown in HUVEC and human retinal microvascular endothelial cells [24]. This is consistent with our findings that STAT3 was the target gene of miR-637. Therefore, we speculated that miR-637 may regulate the proliferation of retinal endothelial cells via targeting STAT3. However, our research also has some limitations. The research population is relatively small, and a larger group is needed to verify the current results. In addition, the specific mechanism of the role of miR-637 in HR function needs further study.

In summary, our research suggests that miR-637 was less expressed in HR patients. MiR-637 is a potential marker for HR patients. miR-637 may participate in the development

of HR patients by acting on the target gene STAT3 to inhibit the proliferation and migration of retinal endothelial cells.

Statement of Ethics

This study was approved by the Research Ethics Committee of Weifang People's Hospital (approval no. 2018437). The research method complies with the standards stipulated in the Declaration of Helsinki. Subjects were informed of the research purpose and experimental procedures before participating and signed the consent form approved by the Ethics Committee.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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There was no funding for this study.

Author Contributions

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript. Weiguang Yang, Manxia Su, Yanli Yu, and Qingxin Fang conducted the experiments. Yusheng Ma supplied critical reagents. Weiguang Yang and Manxia Su wrote the manuscript and Jihong Zhang revised it critically for intellectual content. All authors read and approved the final manuscript.

Data Availability Statement

The corresponding authors may provide data and materials.

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