Original Article
Role of miR-129 in myocardial cells of hypertensive heart disease mouse

Wu Zhu[1], Mei Yu[2], Wanhua Xie[1], Jie Liu[2], Bin Zhao[2]

[1]Clinical Laboratory, Ankang Municipality Of Traditional Chinese Medicine Hospital, Ankang, Shaanxi, China; [2]Ankang Municipality Center Hospital, Ankang, Shaanxi, China

Abstract: Hypertension is one of the most important risk factors of cardiovascular disease especially heart disease. Previous studies showed that microRNA regulates the growth, proliferation, and survival of myocardial cells, and intracellular signal transduction. Although microRNA129 (miR-129) may be associated with cardiovascular disease, the role of miR-129 in myocardial cells of hypertensive heart disease mouse model remains to be further explored. Hypertensive heart disease mouse model was established. Mice cardiomyocytes were isolated and cultured from both hypertensive heart disease mouse and sham-operated mouse using magnetic cell sorting (MACS). RT-PCR was used to detect the levels of miR-129 in cardiomyocytes of both both hypertensive heart disease mouse and sham-operated mouse. Flow cytometry and MTT assay were used to measure cardiomyocytes' growth and survival. Western blot was used to detect the expression and activation of LAST2, a representative molecule of Hippo pathway which was closely related to heart disease. The level of miR-129 in cardiomyocytes of hypertensive heart disease mouse was significantly higher than level of miR-129 in cardiomyocytes of sham-operated mouse. Cell activity of cardiomyocytes of hypertensive heart disease mouse was significantly lower than that of sham-operated mouse. Apoptosis was found in cardiomyocytes of hypertensive heart disease mouse. The expression level of LAST2 was increased in cardiomyocytes of hypertensive heart disease mouse. Decrease of expression level of LAST2 by siRNA reduced apoptosis of cardiomyocytes of hypertensive heart disease mouse. The level of miR-129 in cardiomyocytes of hypertensive heart disease mouse was significantly higher than that of sham-operated mouse. Transfection of miR-129-induced apoptosis of cardiomyocytes, suggesting that miR-129 may be involved in the pathogenesis of hypertensive heart disease. In summary, the expression levels of miR-129 in hypertensive heart disease mouse were significantly higher than that of sham-operated mouse. Overexpression of miR-129 induced apoptosis of cardiomyocytes suggesting that miR-129 involved in the pathogenesis of hypertensive heart disease.

Keywords: miR-129, hypertensive heart disease, cardiomyocytes, apoptosis, programmed cell death, hypertension, hippo pathway

Introduction

Hypertension is one of the most important risk factor for cardiovascular and cerebrovascular diseases, particularly heart disease [1]. Persistent hypertension can lead to morphological and physiological changes of the heart, which if not treatment for a long time could lead to hypertensive heart disease with main symptoms of left ventricular diastolic dysfunction, left ventricular wall thickening, heart failure, and death [2, 3].

The latest research shows that more than 80% of heart failure is closely related, directly or indirectly, to hypertension [4]. When blood pressure rises to a certain extent and maintain for a certain time, coronary heart disease and other complications of heart diseases will show up [5]. Previous studies focused much on pathological fundamental research other than causes of heart disease. Thus, the present study was designed to investigate the role of hypertension on the regulation of myocardial cells on cardiac function [6-8].

Previous studies showed that microRNA regulates the growth, proliferation, survival, mitochondrial morphology, and intracellular signaling of myocardial cells [9, 10]. Although miR-129 may be associated with cardiovascular disease, the role of miR-129 in regulation of

myocardial cells remains to be further explored. Therefore, this study intends to investigate the role of miR-129 in regulation of myocardial cells of hypertensive heart disease mouse model.

Hippo pathway is one of the classic transduction pathways within cardiac cell and plays a key role in the development of tissues and organs [11-13]. Since LAST2 is the most important member of the Hippo, this study focuses mainly on LAST2 molecules. Compared with classic phosphatidylserine signaling pathway, activation of Hippo pathway inhibits cell growth and proliferation, promotes apoptosis and necrosis [14-16]. Studies suggested Hippo pathway may play a role in heart damage and repair process, but the molecular mechanism remains to be further explored. The study will also explore the role of Hippo pathway in the growth and apoptosis of myocardial cells, and the possible molecular mechanisms.

Hypertensive heart disease mouse model in mice was used in this study to investigate the role of miR-129 and Hippo pathway in the growth and apoptosis of cardiomyocytes of hypertensive heart disease mouse and its possible molecular mechanisms.

Materials and experimental methods

Reagents

MTT was purchased from Beijing Dingguo Biological Technology Co., Ltd. FITC-Annexin-V and Caspase-3 activity detection kits were purchased from Biyuntian Biotechnology Institute. DMEM high glucose culture medium, fetal bovine serumm and antibiotics were purchased from Shanghai Biological Engineering Co., Ltd. siRNA of LAST2 and control siRNA were purchased from HaiJima Biological Technology Co., Ltd. Anti-LAST2 monoclonal antibody and internal reference antibody were purchased from sigma. RNA extraction kit and reverse transcription kit were purchased from Shanghai Biological Engineering Co., Ltd.

Construction of hypertensive heart disease mouse model

10-week-old male C57 mice were purchased from Shaanxi Medical University Animal Center and housed in the animal rooms of Shaanxi Medical University. Animal experiments was

reviewed and approved by Ankang Municipality Of Traditional Chinese Medicine Hospital of animal ethics committee. Hypertension mice model was established according to previous study [17]. Specific methods are as follows: electrical pulse was used to stimulate mouse foot at 30 mA for 2 h each day for 8 days. Left anterior descending artery was ligated to cause hypertension. Sphygmomanometer was used to detect blood pressure after the completion of hypertension model. Data showed the success rate was 75% (15/20).

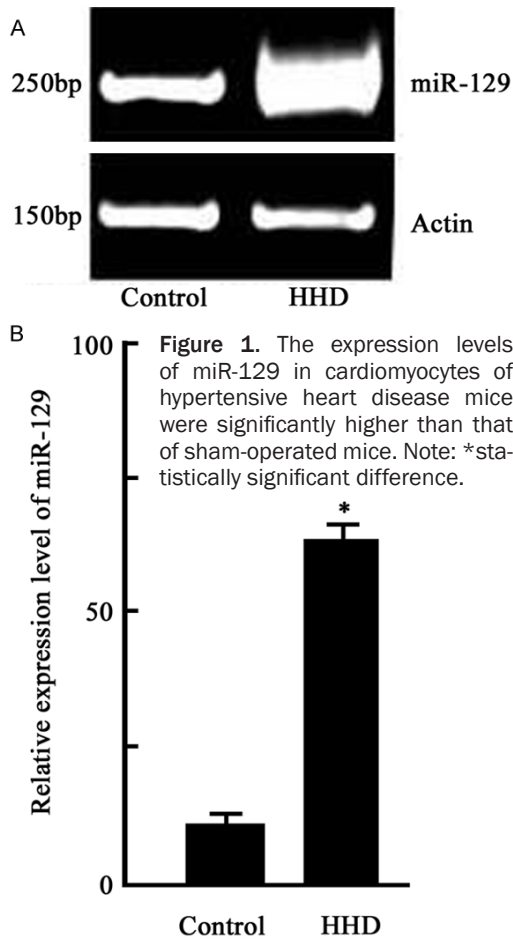### Isolation, purification, and transfection of cardiomyocytes

MACS was used to isolate and culture cardiomyocytes from both hypertensive heart disease (HHD) mouse and sham-operated (Control) mouse [18]. Specifically, mice were anesthetized using chloral hydrate, abdominal skin was opened, heart was removed and cut into pieces using sterilized instruments in a sterile operating room. 1% of Tripsin was added to digest the cells at room temperature for 30 min. Immunomagnetic beads (bead surface coated with specific antibodies against cardiomyocyte specific MG53 monoclonal antibody) were added to cell suspension. PBS was used to wash away non-specific binding. Release buffer was then added to magnetic beads to release cardiomyocytes. The supernatant was collected after centrifugation at 200 rpm, 5 min. Cardiomyocytes were cultured at 37°C with 5% CO_2. The second generation of cardiomyocytes was used in this study.

One day before transfection, cardiomyocytes were inoculated at density of 50%. miR-129 and liposome were mixed and kept at room temperature for 5 min, then added to the cells drop by drop. Medium was changed 12 h later. Cells were cultured for another 24 h for later experiments.

Reverse transcription PCR (RT-PCR)

Cardiomyocytes were isolated and cultured at 37°C with 5% CO_2. Total RNA was extracted using RNA extraction kit according to the manufacturer's instruction, reverse transcribed into cDNA using RT PCR. miR-129 levels in cardiomyocytes of hypertensive heart disease mouse or sham-operated mouse were examined. The primer sequences used in this

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Figure 1. The expression levels of miR-129 in cardiomyocytes of hypertensive heart disease mice were significantly higher than that of sham-operated mice. Note: *statistically significant difference.

study are as follows: 5'-GGCAAATGGCATTCT-GACAT-3' and 5'-AGATGGTGCACGATGCACAG-3', 5'TGTAAAACGACGGCCAGT3' and 5'CAGGAAA-CAGCTATGACC3'.

MTT cell viability assay

Cardiomyocytes were isolated and cultured at 37°C with 5% CO_2. Myocardial cell activity was tested according to the kit instructions. Briefly, the second generation of cardiomyocytes were cultured in 96-well plates. After synchronization pretreatment, 5 ul of MTT was added to each well, mixed, and incubated at room temperature for 30 min. DMSO was used to stop the reaction solution. Finally, the cells were washed with PBS buffer, the absorption values at 490 nm was detected on a microplate reader [20-22].

Myocardial apoptosis detection

Phosphatidylserine overexpression on myocardial cell membrane surface was used to mea-

sure apoptosis using flow cytometry and phosphatidylserine specific dye FITC-annexin [23]. Specific ally, the second generation of cardiomyocytes were digested with 1% tripsin and resuspended in DMEM. FITC-annexin V was added cell suspension and incubated at room temperature for 15 min. Flow cytometry was used to detect the expression of phosphatidylserine on cardiomyocytes membrane surface.
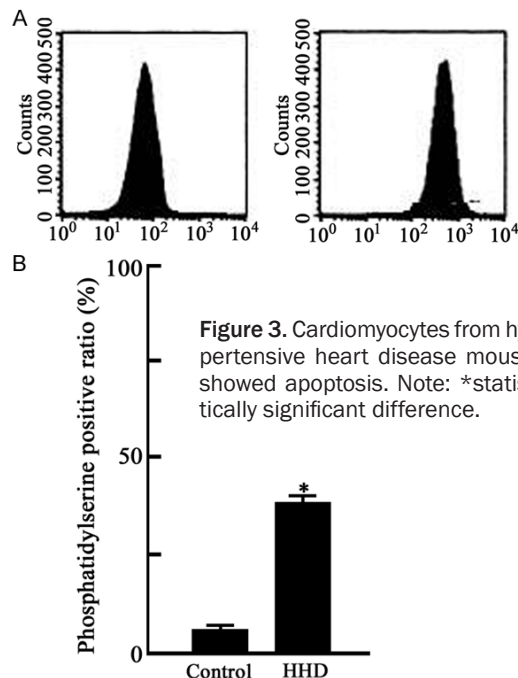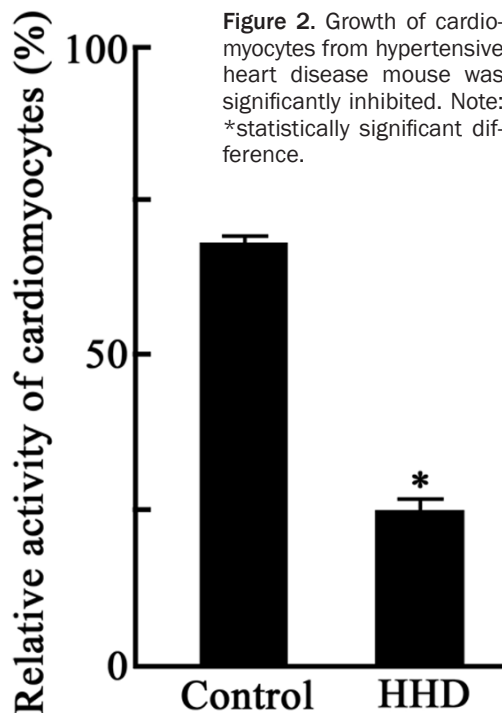
Caspase-3 activity assay

Caspase-3 activity assay was performed according to manufacturer's instructions [24]. Specifically, the second generation of cardiomyocytes were digested with 1% tripsin and centrifuged at 800 rpm for 5 min. Cell pellets were resuspended in DMEM, cells were lysed using lysis buffer, placed on ice for 30 min. Chromogenic substrate of caspase-3 (1 ul) was added to the cell lysate and incubated at room temperature for 15 min. Absorbance values at 490 nm were recorded in a microplate reader.

Western blot

Western blot was used to detect the activation of Hippo pathway [25]. Specifically, ardiomyocytes were digested with 1% tripsin and centrifuged at 800 rpm for 5 min. Cell pellets were resuspended in DMEM, cells were lysed using lysis buffer, placed on ice for 30 min. Cell lysate was loaded to SDS-PAGE. Protein was transferred to PVDF membrane. Membranes were blocked with 5% skim milk for 1 h, washed with TBST, and incubated with rabbit anti-mouse LAST2, Actin antibodies (1:1000) at 4°C overnight. Membranes were washed with TBST, incubated with horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (1:1000) at room temperature for 1 h, washed with TBST, incubated with goat anti-rabbit secondary antibody for 2 hours at room temperature, followed by developing and fixing. Activation of Hippo pathway was analyzed.

Data analysis

Data was analyzed using SPSS 17.0. All data were expressed as mean ± standard deviation (x ± SD). T-test analysis was used to do comparison. Differences between groups were considered significant at P<0.05.

Figure 2. Growth of cardio-myocytes from hypertensive heart disease mouse was significantly inhibited. Note: *statistically significant difference.

Figure 3. Cardiomyocytes from hypertensive heart disease mouse showed apoptosis. Note: *statistically significant difference.

Figure 4. Cardiomyocytes from hypertensive heart disease mouse showed apoptosis. Note: *statistically significant difference.

Results

The expression levels of miR-129 in cardiomyocytes of hypertensive heart disease mice were significantly higher than that of sham-operated mice

The expression levels of miR-129 in cardiomyocytes of both hypertensive heart disease mice and sham-operated mice were measured using RT-PCR. As shown in **Figure 1**, RT-PCR results showed that the expression level of miR-129 in cardiomyocytes of hypertensive heart disease mouse muscle was significantly higher than the level of miR-129 in cardiomyocytes of sham-operated mice.

Growth of cardiomyocytes from hypertensive heart disease mouse was significantly inhibited

This study further explored the role of miR-129 in the pathogenesis of hypertensive heart disease. Cardiomyocytes were first isolated from both hypertensive heart disease mice and sham-operated mice, MTT assay was used to measure the activity of cardiomyocytes.

As shown in **Figure 2**, significant difference was found in the activity of cardiomyocytes
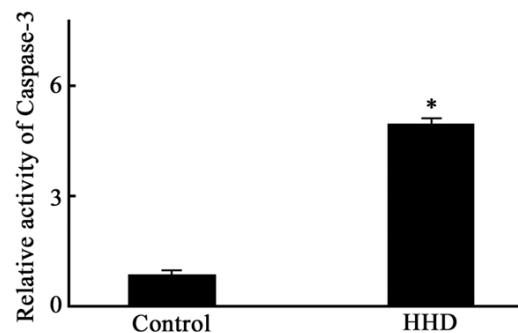
from the two groups. Cardiomyocytes from hypertensive heart disease mouse grew much slower than that of sham-operated mice.

Cardiomyocytes from hypertensive heart disease mouse showed apoptosis

Flow cytometry resuts (**Figure 3**) and and caspase-3 activity results (**Figure 4**) showed that the expression level of cell surface phosphatidylserine of cardiomyocytes from hypertensive heart disease mouse was significantly higher than the sham-operated mice. Moreover, the expression level of activated caspase-3 of cardiomyocytes from hyperten-
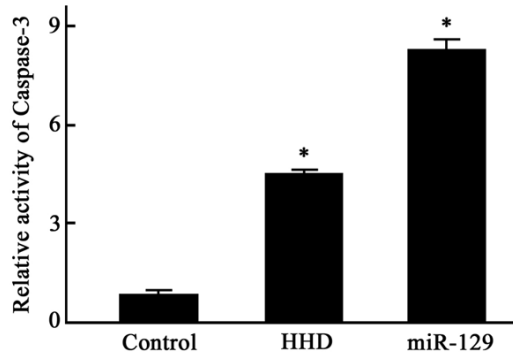
Figure 5. Transfection of miR-129 enhanced cardiomyocyte apoptosis. Note: *statistically significant difference.
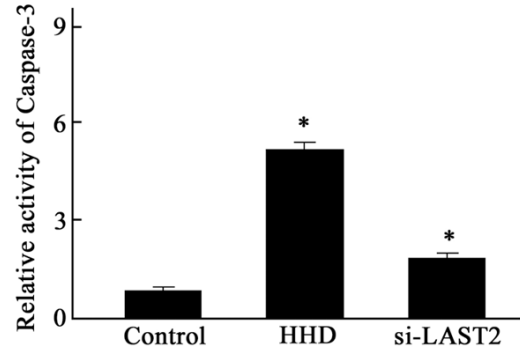

Figure 7. Silence of LAST2 inhibited apoptosis of cardiomyocytes from hypertensive heart disease mice. Note: *statistically significant difference.
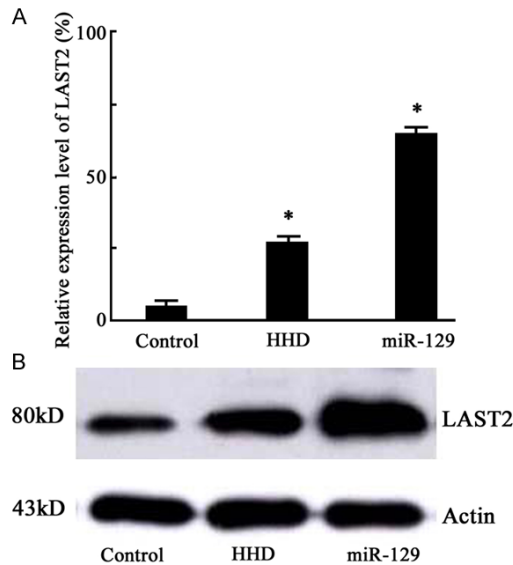

Figure 6. Hippo pathway was activated in cardiomyocytes of hypertensive heart disease mice. Note: *statistically significant difference.

sive heart disease mouse was significantly higher than the sham-operated mice, suggesting that cardiomyocytes from hypertensive heart disease mouse had higher apoptosis level.

Transfection of miR-129 enhanced cardiomyocyte apoptosis

As shown in **Figure 5**, transfection of miR-129 in mouse cardiomyocytes enhanced the activity of caspase-3.

After hypertensive heart disease model, overexpression of miR-129 was overexpressed in cardiomyocyte of hypertensive heart disease

mouse. Overexpression of miR-129 resulted in enhanced cardiomyocyte apoptosis, suggesting the apoptosis seen in cardiomyocyte of hypertensive heart disease mouse was mediated by miR-129.

Hippo pathway was activated in cardiomyocytes of hypertensive heart disease mice

The western blot data (**Figure 6**) showed that compared with sham-operated group, expression level of LAST2, a member of Hippo pathway, in cardiomyocytes from hypertensive heart disease mice was significantly increased. Transfection of miR-129 also resulted in an increase of LAST2 expression

Silence of LAST2 inhibited apoptosis of cardiomyocytes from hypertensive heart disease mice

Figure 7 showed that silence of LAST-2 resulted in the inhibition of apoptosis of cardiomyocytes from hypertensive heart disease mice, indicating that Hippo pathway involved in the apoptosis of cardiomyocytes from hypertensive heart disease mice.

Discussion

In this study, possible regulatory mechanism of miR-129 in mice hypertensive heart disease was investigated. The expression level of miR-129 in cardiomyocytes from hypertensive heart disease mouse was significantly higher than that of sham-operated mice, suggesting that miR-129 may play a role in the regulation of cardiomyocytes from hypertensive heart disease mouse. The present study

further showed that the activity of cardiomyocytes from hypertensive heart disease mouse was lower than that of sham-operated mouse, however, the apoptosis of ardiomyocytes from hypertensive heart disease mouse was significantly higher than that of sham-operated mice. This study further explored the causal link between cardiomyocytes apoptosis and miR-129. Results showed that transfection of miR-129 resulted in enhanced apoptosis in cardiomyocytes from hypertensive heart disease mouse, indicating that miR-129 mediated the apoptosis of cardiomyocytes from hypertensive heart disease mouse [26-28].

Compared with previous studies, this study showed for the first time that the expression level of miR-129 in cardiomyocytes from hypertensive heart disease mouse was significantly higher than that of sham-operated mouse. This study also found that the activity of cardiomyocytes from hypertensive heart disease mouse was significantly lower than that of sham-operated mouse, however, the apoptosis of cardiomyocytes from hypertensive heart disease mouse was significantly enhanced than that of sham-operated mouse. Transfection of miR-129 resulted in apoptosis of cardiomyocytes from hypertensive heart disease mouse, which was in consistent with previous study that miRNA regulates cell growth and apoptosis. Thirdly, the study showed that the expression level of LAST2 was enhanced in cardiomyocytes from hypertensive heart disease mouse. Silencing LAST2 resulted in reduction of apoptosis of cardiomyocytes from hypertensive heart disease mouse, which was in consistent with other study [27].

This study further explored the role of miR-129 in apoptosis of cardiomyocytes from hypertensive heart disease mouse. Various signaling pathways were studied in a pilot experiment including phosphatidylcholine signal pathway, p38 pathway, JNK pathway and Hippo pathway (data not shown). Different from previous studies [26-28], phosphatidylcholine signal pathway, p38 pathway, or JNK pathway was not activated either by overexpression of miR-129 or hypertensive heart disease, suggesting that there might be differences between miR-129 mediated apoptosis of cardiomyocytes from hypertensive

heart disease mouse and classical apoptotic signaling pathways. miR-129 induces apoptosis of cardiomyocytes from hypertensive heart disease mouse via Hippo pathway.

The number of animals (3 in each group) used in this study was a little small, more animals are needed to confirm the reliability of the results. How did miR-129 activate Hippo pathway remains to be elucidated? This study did not collect specimen from patients with hypertensive heart disease to verify the expression level of miR-129, cardiomyocytes apoptosis, and the Hippo pathway activation.

In conclusion, the expression level of miR-129 in cardiomyocytes of hypertensive heart disease mouse was significantly higher than that of sham-operated mouse. Transfection of miR-129 resulted in apoptosis of cardiomyocytes of hypertensive heart disease mouse, suggesting that miR-129 may be involved in the pathogenesis of hypertensive heart disease mouse.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bin Zhao, Shaanxi Ankang Municipality Hospital, 85# South Jinzhou Road, Ankang 72500, Shaanxi, China. Tel: +86-0915-3284013; Fax: +86-0915-3284013; E-mail: rongcui567@163.com

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