Original Article Short-term vagal nerve stimulation improves chronic heart failure via miR-133a-3p upregulation in a rat model

Shuang-Shuang Liu¹, Yan-Hua Xuan¹, Yan Li², Jing Dong¹, Jia-Ying Luo¹, Zhi-Jun Sun¹

Departments of ¹Cardiology, ²Geriatrics, Shengjing Hospital of China Medical University, Shenyang, Liaoning, P. R. China

Received October 4, 2016; Accepted November 30, 2016; Epub January 1, 2017; Published January 15, 2017

Abstract: Background and aim: Autonomic nervous system imbalance is characterized by sympathetic activation and vagal withdrawal, which is an important contributor to the progression of heart failure (HF). We aim to investigate the effect of short-term stimulation on HF and explore the role of miRNAs in this process. Methods: Rats were randomly divided into control (CON), chronic heart failure (CHF), and vagal nerve stimulation (VNS) groups. Rats in the VNS group were subjected to continuous electronic stimulation on the right cervical vagal nerve for 72 hours. The LV function was tested by echocardiography. Furthermore, miRNA expression was assessed by miRNA microarray for randomly selected heart samples from different groups. Finally, miR-133a-3p was selected to further examine its role in myocardial apoptosis. Results: Compared with the CHF group, rats in the VNS group had significantly better LV function, including higher LVEF, lower EDV and ESV. Meanwhile, BNP was decreased in the VNS group (P < 0.05). In both comparisons of CON versus CHF and CHF versus VNS, 11 miRNAs showed significantly altered expression. Indeed, differential expression of three of four selected miRNAs, miR-133a-3p, miR-205, and miR-138-1-3p, was validated using qRT-PCR. We further found that miR-133a-3p could inhibit myocardial apoptosis *in vitro*. Conclusion: Short term vagal nerve stimulation in HF could improve LV function and serum BNP levels in CHF rats, possibly through upregulation of miR-133a-3p, which could inhibit myocardial apoptosis.

Keywords: Apoptosis, electrical stimulation, heart failure, miRNA, vagal nerve

Introduction

Autonomic nervous system imbalance is characterized by sympathetic activation and vagal withdrawal, which is an important contributor to the progression of heart failure (HF) [1, 2]. In clinical studies, an implanted vagal nerve stimulator (VNS) can significantly improve the patient clinical parameters like NYHA class, 6 minute walk test, LV ejection fraction, and LV systolic volumes [3]. VNS is also known to suppress arrhythmias in conscious rats with chronic HF secondary to myocardial infarction [4]. However, it is not known whether a short-term stimulus could have a therapeutic effect on chronic heart.

MiRNAs regulate gene expression by binding to partially complementary sequences in their target mRNAs [5, 6]. Suppression of the miRNA pathway in cardiac muscles leads to heart failure and cardiomyopathy [7]. Some of the miR-NAs are known to regulate cardiac hypertrophy [8], cardiomyocyte proliferation [9], and even induce cardiac regeneration [10].

This study aimed to determine whether shortterm vagal stimulation improves chronic ischemia heart failure. Moreover, we also screened the miRNAs that might be involved in this process to further investigate the disease mechanism.

Materials and methods

Animals and grouping

The experiments were performed on Wistar rats (male, body weight 270-310 g) purchased from the Animal Center of the Chinese Academy of

Medical science, Beijing, China. The current study was approved by the Animal Research Ethics Committee of Shengjing Hospital of China Medical University and were performed in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China. The rats were assigned to the CHF group (chronic heart failure rats treated with sham stimulation; n = 8), VNS group (chronic heart failure rats treated with vagal nerve stimulation; n = 8), and CON group (shamoperated rats treated with sham stimulation; n = 8).

Creation of MI and HF

All rats were subjected to thoracotomy. After intraperitoneal injection with 10% chloral hydrate (0.3 ml/100 g weight), the rats were intubated and connected to a ventilator (HX-300S Animal Respirator, Chengdu Tme Technology Co., Ltd., China). In the CHF group, a 6-0 surgical thread in a curved needle was threaded between the left atrial appendage and the pulmonary cone, then about 2-3 mm from the aortic root at threading to ligate the left anterior coronary artery. In the control group, a simple puncture was made, without ligation at the same site in the heart. The animals were subsequently monitored for 1 to 2 hours for any sign of arrhythmia. Myocardial infarction was confirmed in all animals by the appearance of acute ST-segments and T-wave changes on a limb leads ECG and new anterior wall motion abnormalities on a transthoracic echocardiogram. All the animals were reared to 8 weeks, and transthoracic echocardiographic tests were then performed to assess cardiac function.

VNS treatment

After 8 weeks of myocardial infarction, animals with declined LVEF (< 50%) were randomly separated into heart failure (HF) and VNS groups. After the rats were anesthetized, the right cervical vagal nerve was isolated and an electrode was fixed to it with tissue glue. The electrode went out from the back of the head through the skin and the other end was connected to an electrode stimulator (BL-420S Data Acquisition & Analysis System, Chengdu Tme Technology Co, Ltd., China). The eight rats in the heart failure group were connected to the electrode, but were not given electronic stimulation. The eight rats in VNS group were subjected to continuous electronic stimulation (1 Hz, pulse width 40 ms, strength 5 V). The ECG monitor showed that the heart rate was decreased by 10-20 bpm. After 72 hours of continuous electronic stimulation, transthoracic echocardiography was performed to evaluate cardiac function and blood and tissue samples were collected.

Echocardiography

Transthoracic 2-dimensional echocardiography was performed at 8 weeks after myocardial infarction and 72 hours of VNS treatment. Standard 2-dimensional short-and longparasternal views, as well as 4-chamber, and 3chamber apical views were obtained in a standard manner using an ultrasound probe connected to an echocardiographic machine (PHILIPS iE33, Netherlands) with a S12-4 transducer. The left ventricular end-diastolic, endsystolic volume, and ejection fraction were measured using the Teichholtz method. All volumes were measured in triplicate and their averages were reported. The ultrasound was performed blindly.

BNP assay

Venous blood samples from the CHF and VNS groups were collected in serum separator tubes at the 8-week time point and 72 hours later. Serum was separated after centrifugation at 4500 rpm for 15 minutes in a refrigerated centrifuge at 4°C. The serum was stored at -80°C until required. Serum BNP levels were measured using a human/Mouse/Rat BNP Enzyme Immunoassay Kit (RayBiotech; USA) following the manufacturer's directions. All the samples and standards were measured in triplicate.

MiRNA microarray

Heart samples were randomly selected from different groups of animals (CON = 3, CHF = 3, VNS = 4). Left ventricular samples were collected from the control group. Samples from both the heart failure and the VNS group were separated from the myocardial infarct border zone. Total RNA was isolated using Trizol (Invitrogen) and miRneasy mini kit (QIAGEN) according to the manufacturer's instructions. RNA quality and quantity was measured with the Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies) and RNA Integrity was determined by gel electrophoresis.

	Gene sequence
miR-205-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGACTC
miR-205-FW	GCGTCCTTCATTCCACCG
miR-138-RT	GTC GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCTGGTG
miR-138-FW	CGCGCGGCTACTTCACAA
miR-133-RT	GTCGTATCCAGTGCAGGFGTCCGAGGTATTCGCACTGGATACGACCAGCTGGT
miR-133-FW	CGCGTTTGGTCCCCTTCA
U6-RT	AAAATATGGAAGGCTTCACAGATTTG
U6-FW	CTCGCTTCGFGCAGCACATATACT
U6-RV	ACGCTTCACGAATTTGCGTGTC
Downstream primer	GTGCAGGGTCCGAGGTATTC

 Table 1. MiRNA specific primers

The miRCURY[™] LNA Array (v.18.0) (Exiqon) contains 3100 capture probes, including all human, mouse, and rat miRNAs annotated in miRBase 18.0, as well as all the viral miRNAs related to these species. After RNA extraction from the samples, the miRCURY[™] Hy3[™]/Hy5[™] Power labeling kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's guidelines for miRNA labeling. After stopping the labeling process, the Hy3[™]-labeled samples were hybridized on the miRCURY[™] LNA Array (v.18.0) (Exiqon) as per the array manual.

Scanned images were then imported into the GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. The replicated miR-NAs were averaged and the miRNAs with intensities \geq 30 from all samples were chosen for calculating the normalization factor. The expressed data were normalized using median normalization. After normalization, the differentially expressed miRNAs with statistical significance were identified through Volcano Plot filtering. Hierarchical clustering was performed using MEV software (v4.8, TIGR).

Quantitative analysis of miRNAs

We use qRT-PCR to validate the microarray data. The extracted RNA (500 ng) containing the miRNAs was reverse transcribed to cDNA using a miRNA specific RT primer (50 pmol) using a stem-loop method, at 37° C for 15 minutes, 85° C for 5 seconds, and 4° C. (The miRNA specific RT primer is shown in **Table 1**). The procedure was performed as per the instructions of the PrimeScript RT reagent kit (Takara) in a 20 µl reaction. Diluted cDNA was subjected to qRT-PCR using a SYBR Premix Ex Taq kit (Takara) in a 20 µl reaction containing 1 µl

cDNA, 0.4 μ M of each primer, and 10 μ I of 2*SYBR Green Master Mix, brought to the final volume with RNase-free water. Amplification was performed using the Lightcycler 480 system (Roche) according to the program suggested by kit: 5 minutes preincubation at 95°C, and 40 cycles of 5 seconds of denaturation at 95°C and 30 seconds of amplification at 60°C. No signal was detected in the negative control (no reverse transcriptase or template added). U6 RAN was used as the endogenous control. PCR was performed in triplicate with each sample for both the U6 control and the miRNAs simultaneously. The relative miRNA expression was calculated by the 2^{-ΔΔCT} method.

Cell lines and cell culture

The human myocardial cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Bioind, Israel) and 1% penicillin/streptomycin sulfate. Cells were incubated in a humidified incubator supplemented with 5% CO₂ at 37°C.

Plasmid transfection

Hsa-miR-133a-3p mimics (pGCMV/EGFP) and empty vector (NC) were synthesized by Gene-Pharma (Shanghai, China). Plasmids were transfected into subconfluent cells according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen-Life Technologies, CA) (DNA/Lipofectamine 2000 = 1/2.5). Stably transfected cell lines were established after the cells were incubated in complete DMEM with blasticidin ($12 \mu g/mL$) (Sigma, Shanghai, China) or G418 (500 mg/ml) (Sigma, Shanghai, China) for 15 days. We verified the clones using real-time quantitative polymerase chain reaction (RT-PCR) and pooled the positive clones for further experiments.

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay was used for cell proliferation analysis following the manufacturer's instructions. HCM cells with established stable expression (NC, miR-133a-3p mimics) were seeded at a density of 5×10^3 cells per well in 96-well plates and were incubated for various periods (0, 24, 48, and 72 hours). Absorbance at 450 nm was measured using an electroluminescence immunosorbent assay reader (Thermo Fisher Scientific, Waltham, MA).

Flow cytometry analysis of apoptosis

Cells were collected and washed twice with cold phosphate-buffered saline solution (PBS) to remove the floating cells before analysis with the Annexin V-APC Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China). Apoptosis was evaluated using a flow cytometry analyzer (BD Biosciences, San Jose, CA). Data were analyzed using the BD Accuri C6 software.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling assay

The Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) (Roche, Shanghai, China) assay has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis [18]. Cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with the in situ cell death detection reagents from Roche at 37°C for 1 hour. The samples were then mounted in mounting media containing DAPI. Fluorescent images were captured using a fluorescence microscope at 20× magnification. The total number of DAPI positive cells and TUNEL positive cells were counted from at least five images from each sample.

Caspase-3 activity analysis for apoptosis

Caspase-3 activity was determined using the caspase-3 colorimetric activity assay kit (Beyo-time, Shanghai, China) according to the manu-

facturer's instructions. Briefly, cells were collected, washed twice with cold PBS, and lysed with the cell lysis buffer included in the kit. Equal amounts of protein were incubated with Ac-DEVD-AMC, a caspase-3 substrate, at 37°C for 2 hours. Caspase-3 activity was determined by measuring absorbance at 405 nm using an electroluminescence immunosorbent assay reader.

Isolation of RNA and quantitative polymerase chain reaction analysis

Total RNA was extracted from cultured cells using Trizol (Invitrogen, Shanghai, China) following the manufacturer's protocols, and reverse transcribed using miRNA-specific RT primers (RiboBio, Guangzhou, China) for miR-33a-3p. Reverse-transcribed cDNA was measured by quantitative polymerase chain reaction (qPCR) using a SYBR Green PCR Kit (QIAGEN, Shanghai, China) on an Applied Biosystems 7500 Fast Sequence Detection System under the following conditions: denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing and extension at 60°C for 30 seconds. The relative miRNA and mRNA expression levels were normalized to U6 and β -actin, respectively.

Western blotting

Cells were lysed in the presence of a protease and phosphatase inhibitor mixture (3 µL) and were then homogenized. The supernatant fraction was collected and its protein concentration was determined with a bicinchoninic acid protein assay kit (Beyotime, Hangzhou, China). An aliquot containing 20 µg of denatured protein from each sample was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and incubated overnight with primary antibody (1:1000 dilution) at 4°C, followed by incubation with the secondary antibody, rabbit horseradish peroxidase-conjugated anti-goat IgG (1:2000 dilution; both, Cell Signaling Technology, Boston, MA) for 1 hour at ambient temperature. The blots were then incubated with enhanced chemiluminescence solution for 1 minute. The emitted signals were detected by the ChemiDoc XRS+ Chemiluminescence imaging system (Bio-Rad, Hercules, CA) and processed using the Image Lab Software



Figure 1. LV function of the rats as tested by echocardiography. A: EDV of the rats in each group; B: LVESV of the rats in each group; C: LVEF of the rats in each group. EDV: End-Diastolic Volume; LVESV: Left Ventricular End Systolic Volume; LVEF: Left Ventricular Ejection Fraction. *: P < 0.05.



Figure 2. Serum BNP levels in rats of the CHF and VNS groups. *: P < 0.05.

(both, Bio-Rad, Inc., Hercules, CA, USA). The gray value of each band in the imaging data was analyzed using the Quantity One software (Bio-Rad, Inc. CA).

Statistical analysis

Data were expressed as mean \pm SD wherever appropriate. Differences between two groups were examined by an independent samples *t*-test. Comparison of cardiac function before and after the VNS treatment was carried out with a paired samples *t*-test. Data analysis was performed with SPSS 19.0. Differences were considered significant at a value of P < 0.05.

Results

Effects of VNS on LV function

Rats from all groups, including control, heart failure, and the VNS group completed the 8-week plus 72-hour experimental period. As

shown in Figure 1, the baseline LV function was compared among the CON, CHF, and VNS group rats. The values of EDV, ESV, and EF showed no significant differences among the three groups at the baseline. At 8 weeks after MI surgery, the LV function of rats from the CHF and VNS groups were obtained. Before vagal nerve stimulation, the LV function of both groups was equal. After the 72 hour stimulation and sham treatment, a significant increase of EF was seen in the VNS group compared with that in the CHF group (54.5 ± 7.5% vs. 42.9 ±

3.1%, P = 0.024). Similar improvements were also observed with EDV and ESV values between the CHF and VNS groups (EDV: 0.95 \pm 0.20 vs. 0.97 \pm 0.41, P = 0.01; ESV: 0.43 \pm 0.11 vs. 0.56 \pm 0.2, P = 0.001).

We also compared the LV ejection fraction before and after the 72-hour VNS treatment in the VNS group. The LV ejection fraction was significantly improved from $44.1 \pm 4.0\%$ to $54.5 \pm 7.5\%$ (P < 0.05). However, a difference in LVEF was not observed in the heart failure group before and after 72 hours.

Effects of VNS on serum BNP levels

At 8 weeks after MI surgery in rats, the serum BNP level in the CHF group was 126.7 ± 18.2 pg/L and that in the VNS group was 131.1 ± 19.8 pg/L (P = 0.655). However, after 72 hours, an obvious difference was seen between the CHF and VNS groups (CHF: 131.1 ± 19.8 pg/L vs. VNS: 113.6 ± 20.7 pg/L, P =





0.041). In order to compare the efficacy of VNS, we used a paired *t*-test on serum BNP values before and after treatment. As shown in **Figure 2**, the serum BNP levels decreased by 16% (P = 0.042).

MiRNA microarray

Samples were randomly selected from three groups for determining the miRNA expression profiles (CON = 3, CHF = 3, VNS = 4). In the CHF group, the expression of 29 miRNAs (**Figure 3**) was increased and that of 12 miRNAs was sup-

pressed compared with that in tissue samples from the same areas in the CON group. Moreover, in the VNS group, the levels of 25 miRNAs (**Figure 4**) were elevated over those in the CHF group, and 22 miRNAs were suppressed.

In both comparisons, 11 miR-NAs showed significantly altered expression. Of these, three miRNAs (rno-miR-205, rno-miR-540-3p, and rno-mi-R-495) were elevated in the CHF group and were reduced in the VNS group. The remaining, eight miRNAs (rno-miR-103-3p, rno-miR-150-5p, rnomiR-350, rno-miR-138-1-3p, rno-miR-3571, rno-miR-129-1-3p, rno-miR-133a-3p, and rno-miR-133b-3p) were suppressed in the CHF group and were increased in the VNS group. Thus, we considered that these miRNAs might be involved in the process of heart failure and its improvement by vagal nerve stimulation.

Real-time qRT-PCR validation

To validate the results of the microarray, we selected four miRNAs as our major study subjects. We chose miR133a-3p because it has been widely reported in both human and animal heart tissue from different CHF models. MiR-138-1-3P showed the high-

est fold-change in two comparisons. Due to the impressive changes in the VNS group compared with those in the CHF group, miR-205 and miR-540-3p were selected.

On qRT-PCR analysis of all tissues from three groups, we compared the expression of miR-NAs between the three groups. As shown in **Figure 5**, other than the reduced expression of miR-540-3p in all tissues, which could not be measured, the qRT-PCR results of miR-133a-3p, miR-205, and miR-138-1-3p were consistent with those of the miRNA microarray.

Short-term vagal nerve stimulation improves chronic heart failure



Figure 4. MiRNA expression spectrum clustering map between the CHF and VNS group. The green bar indicates downregulation, and the red bar indicates upregulation.

MiR-133a-3p overexpression increased the proliferation of HCM cells

As shown in **Figure 6A**, miR-133a-3p expression was significantly increased in HCM cells after treatment with miR-133a-3p mimics (P < 0.001). CCK8 results revealed that ectopic overexpression of miR-133a-3p increased the proliferation of HCM cells at 48 and 72 hours (all panels, P < 0.01), whereas no significant difference was observed at 24 hours in relation to the controls (NC) (P > 0.05) (**Figure 6B**). In addi-tion, we also found that restoration of miR-133a-3p increased the protein expression of PCNA and Ki-67 in HCM cells (**Figure 6C**).

MiR-133-3p overexpression suppressed hypoxia-induced apoptosis in HCM cells

Flow cytometry results showed that both early and late apoptotic populations were increased upon miR-133a-3p overexpression in HCM cells (all panels, P < 0.01) (Figure 7A. 7B). Apoptosis was also measured using the TUNEL assay, and miR-423-5p overexpression could significantly decrease the percentage of TUNEL positive cells compared with those in NC (all panels, P < 0.01) (Figure 8A, 8B). Meanwhile, Caspase-3 activity and protein expression levels of NF-kB, Bcl-2, and Bax in HCM cells were decreased after transfection with miR-133a-3p mimics (all panels, P < 0.01) (Figure 9A, 9B).

Discussion

In this study, we found that continuous short-term VNS treatment could significantly attenuate chronic heart failure in a rat model after myocardial infarction. Moreover, we used the micro-RNA microarray to detect differen-tial miRNA expression in the control, heart failure, and VNS groups. Of the analyzed miR-NAs, three differentially expr-

essed miRNAs were validated by qRT-PCR. The results indicate that miR133a-3p, miR-138-1-3P, and miR-205 may participate in the improvement of heart failure mediated by vagal nerve stimulation. Furthermore, we demonstrated that miR133a-3p could inhibit myocardial apoptosis *in vitro*.

Several studies show that chronic VNS treatment could attenuate heart failure in different phases, and delay the HF process in a high rate ventricular pacing HF model [11]. VNS can also prolong survival time in rat myocardial infarction models [12]. A recently published clinical trial showed that after 6 months of cervi-



Figure 5. The expression of miR-133a-3p, miR-138-1-38, and miR-205 as tested by miRNA microarray and qRT-PCR. A: Expression of miR-133a-3p; B: Expression of miR-138-1-38; C: Expression of miR-205. *: P < 0.05.



Figure 6. MiR-133a-3p overexpression induces the proliferation of HCM cells. A: Expression of miR-133a-3p in HCM cells after transfection with miR-133a-3p mimics. B: HCM cells treated with miR-133a-3p mimics or control mimics were subjected to CCK8 assays. C: Protein levels of PCNA and Ki-67 in HCM cells were measured by western blotting followed by densitometric analysis. *: P < 0.05.



Figure 7. MiR-133a-3p overexpression inhibits hypoxia-induced apoptosis of HCM cells. A: Early apoptotic cells (lower right, LR) and late apoptotic cells (upper right, UR) were shown. B: Histograms represent quantification of the rate of apoptosis. *: P < 0.05.

cal vagal nerve stimulation, NYHA class was significantly improved in HF patients [13]. However, other clinical studies demonstrated negative results [14]. Animal studies and clinical trials were inconsistent in aspects of frequency and duration of electrical stimulation, and in the timing of therapy initiation. In our study, we established a rat model of chronic heart failure after myocardial infarction. We found that short-term vagal nerve stimulation for ischemic heart failure had a therapeutic effect, which was confirmed by echocardiography and biochemical indicators. These stimulation parameters and durations might be useful for clinical trials.

MiRNAs are small 22 nucleotide non-proteincoding RNAs that inhibit transcription or translation by interaction with the 3'-untranslated regions of target mRNA [15]. Because of their



Figure 8. MiR-133a-3p overexpression inhibits hypoxia-induced apoptosis in HCM cells. A: Representative images of the TUNEL assay. Green staining represents TUNEL positive cells, and blue staining represents DAPI positive cells. B: Apoptosis index representing the percentage of TUNEL positive HCM cells.



Figure 9. MiR-133a-3p overexpression inhibits hypoxia-induced apoptosis in HCM cells. A: Histograms represent quantification of caspase-3 activity. B: The protein levels of NF- κ B, Bcl-2, and Bax in HCM cells. APC: Allophycocyanin; PI: propidium iodide. *: P < 0.05.

ability to monitor the expression levels of genes that control both the adaptive and maladaptive cardiac remodeling processes, miRNAs may be vitally involved in the pathogenesis of heart failure [16]. In addition, miRNAs are involved in the regulation of biological processes, which may help direct the future research.

In rats with chronic heart failure, seven miRNAs were downregulated, which were upregulated after vagal nerve stimulation. In contrast, the expression of three miRNAs was increased in the myocardial tissue from animals with heart failure, and was decreased after vagal nerve stimulation. These results indicate that these miRNAs may be involved in the entire process of heart failure development after myocardial infarction, as well as ameliorated heart failure after vagal nerve stimulation. Several miRNAs are reported to be differentially expressed in cardiac hypertrophy, for instance, mir-1, mir-133, mir-29, mir-30, and mir-150 were downregulated whereas mir-23a, mir-24, mir-125, mir-129, mir-195, mir-199, mir-212, and mir-208 were upregulated [17]. Consistently, some of these miRNAs were confirmed in our miRNA array. Thus, our findings suggested that vagal nerve activity affected miRNA maturation. To confirm that these miRNAs are involved in heart failure regulation, we used qRT-PCR to verify the differential expression of selected miRNAs, of which miR-133a, miR-138-1-3p, and miR-205 showed differences in expression (P < 0.05). Alteration of miRNA expression is a complicated network response to regulate multiple gene expression wherein each miRNA may target several mRNAs. As simultaneous changes in several miRNAs were observed in response to vagal nerve stimulation [18], further investigation of these miRNAs is needed to reveal the mechanisms underlying vagal nerve stimulation in heart failure.

MiR-133 was found to be deregulated in human myocardial infarction [19], and miR-133 directly downregulates CTGF, a key profibrotic protein, which plays an important role in controlling structural changes of the myocardial extracellular matrix [20]. MiR-133 showed opposing effects on apoptosis by targeting HSP60. HSP70, and caspase-9 in cardiomyocytes [21]. In particular, overexpression of β1-adrenergic receptors lead to downregulation of miR-133, as observed in mice models of heart failure [22]. Our study confirmed that miR-133a-3p was involved in the entire process from the occurrence of heart failure to the improvement after vagal nerve stimulation. This included a change in the failure to pay the sympathetic development and stimulation of the vagal nerve as a treatment to improve the prognosis of heart failure in two processes. Moreover, we found that overexpression of miR-133a-3p induced proliferation and inhibited hypoxiainduced apoptosis of HCM cells. These results indicated that short-term vagal nerve stimulation improved LV function and serum BNP levels in CHF rats, possibly through upregulation of miR-133a-3p.

In conclusion, continuous short-time vagal nerve stimulation could improve chronic heart failure in a rat model of chronic myocardial ischemia. In addition, mirR-133a-3p, miR-138, and miR-205 might be involved in the mechanism underlying VNS treatment. We further demonstrated the inhibitory role of miR-133a-3p on hypoxia-induced apoptosis in HCM cells.

Acknowledgements

This study was supported by Liaoning Provincial Science and technology Department (201140-4013-7).

Disclosure of conflict of interest

None.

Address correspondence to: Zhi-Jun Sun, Department of Cardiology, Shengjing Hospital of China Medical University, 36 Sanhao Road, Shenyang 110004, Liaoning, P. R. China. Tel: +86-1506897-0690; E-mail: doctorszj123@163.com

References

- Floras JS. Clinical aspects of sympathetic activation and parasympathetic withdrawal in heart failure. J Am Coll Cardiol 1993; 22: 72A-84A.
- [2] Porter TR, Eckberg DL, Fritsch JM, Rea RF, Beightol LA, Schmedtje JF Jr and Mohanty PK. Autonomic pathophysiology in heart failure patients. Sympathetic-cholinergic interrelations. J Clin Invest 1990; 85: 1362-1371.
- [3] De Ferrari GM, Crijns HJ, Borggrefe M, Milasinovic G, Smid J, Zabel M, Gavazzi A, Sanzo A, Dennert R, Kuschyk J, Raspopovic S, Klein H, Swedberg K, Schwartz PJ; CardioFit Multicenter Trial I. Chronic vagus nerve stimulation: a new and promising therapeutic approach for chronic heart failure. Eur Heart J 2011; 32: 847-855.
- [4] Zheng C, Li M, Inagaki M, Kawada T, Sunagawa K and Sugimachi M. Vagal stimulation markedly suppresses arrhythmias in conscious rats with chronic heart failure after myocardial infarction. Conf Proc IEEE Eng Med Biol Soc 2005; 7: 7072-7075.
- [5] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.
- [6] Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 2012; 13: 271-282.
- [7] Rao PK, Toyama Y, Chiang HR, Gupta S, Bauer M, Medvid R, Reinhardt F, Liao R, Krieger M, Jaenisch R, Lodish HF and Blelloch R. Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. Circ Res 2009; 105: 585-594.
- [8] Callis TE, Pandya K, Seok HY, Tang RH, Tatsuguchi M, Huang ZP, Chen JF, Deng Z, Gunn B, Shumate J, Willis MS, Selzman CH and Wang DZ. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J Clin Invest 2009; 119: 2772-2786.
- [9] Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ and Srivastava D. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell 2007; 129: 303-317.

- [10] Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S and Giacca M. Functional screening identifies miRNAs inducing cardiac regeneration. Nature 2012; 492: 376-381.
- [11] Zhang Y, Popovic ZB, Bibevski S, Fakhry I, Sica DA, Van Wagoner DR and Mazgalev TN. Chronic vagus nerve stimulation improves autonomic control and attenuates systemic inflammation and heart failure progression in a canine high-rate pacing model. Circ Heart Fail 2009; 2: 692-699.
- [12] Li M, Zheng C, Sato T, Kawada T, Sugimachi M and Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation 2004; 109: 120-124.
- [13] Premchand RK, Sharma K, Mittal S, Monteiro R, Dixit S, Libbus I, DiCarlo LA, Ardell JL, Rector TS, Amurthur B, KenKnight BH and Anand IS. autonomic regulation therapy via left or right cervical vagus nerve stimulation in patients with chronic heart failure: results of the AN-THEM-HF trial. J Card Fail 2014; 20: 808-816.
- [14] Zannad F, De Ferrari GM, Tuinenburg AE, Wright D, Brugada J, Butter C, Klein H, Stolen C, Meyer S, Stein KM, Ramuzat A, Schubert B, Daum D, Neuzil P, Botman C, Caste MA, D'Onofrio A, Solomon SD, Wold N and Ruble SB. Chronic vagal stimulation for the treatment of low ejection fraction heart failure: results of the neural cardiac therapy for heart failure (NECTAR-HF) randomized controlled trial. Eur Heart J 2015; 36: 425-33.
- [15] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
- [16] Sayed D, Hong C, Chen IY, Lypowy J and Abdellatif M. MicroRNAs play an essential role in the development of cardiac hypertrophy. Circ Res 2007; 100: 416-424.

- [17] Mishra PK, Tyagi N, Kumar M and Tyagi SC. MicroRNAs as a therapeutic target for cardiovascular diseases. J Cell Mol Med 2009; 13: 778-789.
- [18] Boettger T and Braun T. A new level of complexity: the role of microRNAs in cardiovascular development. Circ Res 2012; 110: 1000-1013.
- [19] Bostjancic E, Zidar N, Stajer D and Glavac D. MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction. Cardiology 2010; 115: 163-169.
- [20] Duisters RF, Tijsen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, Herias V, van Leeuwen RE, Schellings MW, Barenbrug P, Maessen JG, Heymans S, Pinto YM and Creemers EE. miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. Circ Res 2009; 104: 170-178, 176p following 178.
- [21] Xu C, Lu Y, Pan Z, Chu W, Luo X, Lin H, Xiao J, Shan H, Wang Z and Yang B. The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes. J Cell Sci 2007; 120: 3045-3052.
- [22] Dockstader K, Nunley K, Karimpour-Fard A, Medway A, Nelson P, Port JD, Liggett SB, Bristow MR and Sucharov CC. Temporal analysis of mRNA and miRNA expression in transgenic mice overexpressing Arg-and Gly389 polymorphic variants of the beta1-adrenergic receptor. Physiol Genomics 2011; 43: 1294-1306.