Upregulation of miR-122 is associated with cardiomyocyte apoptosis in atrial fibrillation

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Abstract. Atrial fibrillation (AF) is a common cardiac arrhythmia, which is associated with increased cardiovascular morbidity and mortality. microRNA (miRNA/miR)-122 has been reported to be related with heart diseases, however, the functional role of miR-122 in atrial fibrillation is unclear. Therefore, the aim of the present study was to investigate the roles of miR-122 in atrial fibrillation. Male C57BL/6 mice were divided into the following three groups: Control, sham-operation and AF. Mice in the AF group received transesophageal rapid atrial stimulation for the induction of AF. Cardiomyocytes isolated from mice in the AF group and were transfected with miR-122 inhibitors. Reverse transcription-quantitative polymerase chain reaction was used to assess the expression of miR-122 in cardiomyocytes isolated from mice in the AF, sham-operation and control groups, and in cells transfected with miR-122 inhibitors. MTT and TUNEL assays were used to evaluate cardiomyocyte viability and apoptosis, respectively. Western blot analysis was used to assess the expression levels of extracellular signal-regulated kinase (ERK) and phosphorylated (p)-ERK, as well as the apoptosis-associated proteins caspase-3 and B-cell lymphoma 2-like 1 (Bcl-x). The present results demonstrated that miR-122 expression in the AF group was significantly increased compared with the sham-operation and control groups, whereas it was significantly decreased following transfection with the miR-122 inhibitor. Cardiomyocyte viability was increased and their apoptosis rate was significantly decreased following miR-122 transfection. In addition, the expression of the anti-apoptotic protein Bcl-x was significantly upregulated, whereas the expression of the pro-apoptotic caspase-3 was significantly downregulated following miR-122 inhibition. Furthermore, the p-ERK/total ERK ratio was significantly increased in the miR-122 inhibitor

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group compared with the AF and control groups. The present results suggested that miR-122 may be implicated in the molecular mechanisms underlying the proliferation and apoptosis of cardiomyocytes in AF.

Introduction

Atrial fibrillation (AF) is an abnormal heart rhythm characterized by rapid and irregular beating, which has a prevalence of ~1% in the general population and >8% in patients aged ≥80 years old (1,2). AF is not a life-threatening arrhythmia; however, it impairs the quality of life of patients as a result of its anatomic, hemodynamic, and hemocoagulative consequences (3). AF markedly influences the morbidity and mortality of patients (4), as it can increase the risk of heart failure and stroke (5). In addition, as its prevalence continuously increases, AF has become an important cause of healthcare expenditure worldwide (6,7). Therefore, the elucidation of the molecular mechanisms underlying the pathophysiology of AF is of critical importance.

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNA molecules, with a length of 18-22 nucleotides (8). miRNAs bind to the 3'-untranslated region of target mRNAs and thus regulate the expression of numerous target genes (9); miRNAs have been reported to regulate ~60% of genes (10). Aberrant miRNA expression has been implicated in various pathophysiological processes, including the pathogenesis of cardiac diseases, and their detection may have potential as a novel diagnostic tool (11). Several miRNAs have been reported to be abnormally expressed in AF, including miRNA (miR)-328 (12), miR-1 (13) and miR-26 (14). The expression of miR-122 has previously been associated with the development of cardiovascular diseases, including acute coronary syndrome (15) and myocardial fibrosis (16). In addition, Liu et al (9) demonstrated that serum miR-122 levels were significantly decreased in rats with experimental stroke produced by middle cerebral artery occlusion, as well as in humans with ischemic stroke. Therefore, it is hypothesized that miR-122 may be implicated in the development and progression of AF.

In the present study, an AF mouse model was generated by transesophageal rapid atrial stimulation. Cardiomyocytes were isolated from AF mice, transfected with miR-122 inhibitors or negative controls and miR-122 expression was assessed. In addition, cardiomyocyte proliferation and apoptosis were evaluated. In order to investigate the molecular mechanisms underlying the effect of miR-122 in AF, western blot analysis was used to assess the expression of extracellular signal-regulated kinase (ERK) and phosphorylated (p)-ERK, as well as of the apoptosis-associated proteins caspase-3 and B-cell lymphoma 2-like 1 (Bcl-x). The present study aimed to provide evidence suggesting the implication of miR-122 in the mechanisms underlying AF pathophysiology.

Materials and methods

Animals. Male C57BL/6 mice were originally purchased from Japan SLC, Inc. (Shizuoka, Japan), and were provided with standard food and water. In the present study, 30 male mice (age, 12-14 weeks; weigh, 22-24 g) were used. The mice were housed in a pathogen-free facility with free access to standard food and water. The temperature was maintained at $25\pm2^{\circ}$ C and the relative humidity was $55\pm10\%$ under a 12 h light/12 h dark. All experimental procedures were approved by the Ethics Committee of Tianjin Medical University General Hospital (Tianjin, China).

AF model. A total of 30 male C57BL/6 mice were divided into the following three groups (n=10 mice/group): AF, sham-operation and control group. Transesophageal rapid atrial stimulation was used for the induction of AF, as previously described (17). Briefly, mice were anesthetized by isoflurane inhalation (1.5-2% for maintenance). A 1.1 French octapolar catheter with eight circular electrodes (0.5 mm) and an interelectrode distance of 1 mm (cat no. EPR 800; Millar, Inc., Houston, TX, USA) was advanced through the esophagus. The catheter was placed at the site with the lowest threshold for atrial capture, as previously described (18). In order to ensure the correct position of the pacing catheter, atrial capture with 1:1 atrioventricular conduction was measured prior to the burst pacing period (19). Transesophageal atrial burst pacing was then induced for 10 sec, at a stimulation amplitude of 1.5 mA, using 10 msec cycle lengths and a pulse width of 3 mA. Mice in the sham-operation group did not receive transesophageal atrial burst pacing. The mice in the control group received no intervention.

Cell isolation and culture. Two weeks following operation, the cardiomyocytes were isolated from all the three groups as previously described (20). Following 3-5 days of culture, cardiomyocytes that exhibited regular spontaneous contractions were used for experiments. Cardiomyocytes were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal calf serum (FCS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 0.08 mg/ml gentamicin and maintained in 95% air and 5% CO₂ at 37°C.

Cell transfection. miR-122 inhibitors and inhibitor controls were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China).miRNAs (100 nM) were transiently transfected into target cells from AF mice (1x10⁴ cells) using Lipofectamine 2000[®] (Invitrogen; Thermo Fisher Scientific, Inc.) as the transfection reagent, according to the manufacturer's protocol. Following 48 h of transfection, subsequent experiments were performed. The sequences for miR-122 inhibitor was 5'-CAAACACCA UUGUCACACUCCA-3' and control was 5'-GCCCAGGCA AAUGACACAGUUC-3'.

MTT assay. Cells were seeded in 96-well plates at a density of 10³ cells/well. Cell viability was assessed following 72 h and 7 days of culture using an MTT colorimetric assay, according to the manufacturer's protocol. Briefly, 10 μ l (5 mg/ml) MTT solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well. Following 4 h of incubation at 37°C, the samples were centrifuged at 4°C and 600 x g for 5 min, and the formazan product was dissolved with DMSO. The optical density of the supernatants was measured at 570 nm using a UV-Vis microplate spectrophotometer (BioTek China, Beijing, China). The experiment was repeated three times.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay. Cardiomyocyte apoptosis was assessed using the TUNEL assay kit (Roche Diagnostics, Indianapolis, USA). Apoptotic nuclei were fluorescently labeled with the 50 μ l TUNEL reagent for 1 h at 37°C in the dark. Cell nuclei were counterstained with 10 μ g/ml Hoechst 33258 for 5 min at room temperature. The number of apoptotic cells was determined under a confocal fluorescence microscope (Olympus, Otsu, Japan). The apoptotic cells were calculated as the number of TUNEL-positive and DAPI-stained cells divided by the total number of cells in five random fields. This assay was repeated at least three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using an RT Reagent kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. qPCR was performed using the SYBR-Green PCR Master Mix (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Thermocycling conditions were as follows: Denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. The primers for miR-122 (cat no. 3416) and small nucleolar RNA D68 (Snord68; cat no. 33712), used as the housekeeping gene, were synthesized by Qiagen GmbH (Hilden, Germany). Relative miRNA expression was quantified using the $2^{-\Delta\Delta Cq}$ method (20). The primers were listed as follows: miR-122 forward, 5'-GAGAGGCCTAAAGCCACAGA-3' and reverse, 5'-CACTTACCCCCAGTCAGCTC-3'. Snord68 forward, 5'-ACGACTAGGGCTGTACTGACTTGATG-3' and reverse, 5'-CTCAACTGGTGTCGTGGAGTCGG-3'.

Western blot analysis. Cardiomyocytes were lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with a cocktail of protease and phosphatase inhibitors on an ice bath. Following ultrasonic fragmentation, lysates were centrifuged at 1,500 x g for 30 min at 4°C, and the supernatant was collected into an Eppendorf tube. Protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts (20 μ g) of extracted protein



Figure 1. miR-122 expression is upregulated in the cardiomyocytes of AF mice. Reverse transcription-quantitative polymerase chain reaction was used to assess miR-122 expression levels in cardiomyocytes from control, sham-operation, AF, miR-122 inhibitor and miR-control groups. Data are expressed as the mean \pm standard deviation and the experiments were repeated at least three times. *P<0.05 vs. control; #P<0.05 vs. AF. AF, atrial fibrillation; miR, microRNA.



Figure 2. Cardiomyocyte viability was evaluated using an MTT assay. Cardiomyocytes were isolated from AF mice and transfected with either a miR-control or miR-122 inhibitor. Untransfected AF cells were used as a control. Data are expressed as the mean \pm standard deviation and the experiments were repeated at least three times. *P<0.05 vs. AF. AF, atrial fibrillation; miR, microRNA.

samples were separated by 10-12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.). Membranes were blocked with 5% nonfat dry milk at room temperature for 1 h, and then probed with anti-Bcl-x (cat. no. 2764; dilution 1:1,000), anti-caspase-3 (cat. no. 9662; dilution 1:1,000), anti-ERK (cat. no. 4695; dilution 1:1,000) and anti-p-ERK (cat. no. 4370; dilution 1:1,000) primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. Following three washes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; dilution 1:2,000) at room temperature for 1 h (Cell Signaling Technology, Inc.) for 2 h. β-actin (cat. no. 4970; dilution 1:1,000) was used as the internal control (Cell Signaling Technology, Inc.). Protein bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences; GE Healthcare, Chicago, IL, USA). Blots were semi-quantified by densitometric analysis on a gel documentation system using the Image Lab Software version 5.2.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Enumerated data were analyzed using a χ^2 or Wilcoxon rank-sum test. The statistical significance of the differences between groups was assessed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. Data are expressed as the mean \pm standard deviation and the experiments were repeated at least three times. Statistical analysis was performed using the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-122 expression in cardiomyocytes. RT-qPCR was used to assess the expression of miR-122 in cardiomyocytes (Fig. 1). The present results demonstrated that the expression of miR-122 in the AF group was significantly increased compared with the sham-operation and control groups (P<0.05). By contrast, following transfection with a miR-122 inhibitor, the expression of miR-122 in cardiomyocytes isolated from AF mice was significantly decreased compared with AF cardiomyocytes transfected with a miR-control (P<0.05).

Cardiomyocyte viability. Cardiomyocytes isolated from AF mice were transfected with a miR-122 inhibitor or an inhibitor control, and cell viability was evaluated 72 h and 7 days post-transfection, using an MTT assay (Fig. 2). Following miR-122 inhibition, the viability of cardiomyocytes appeared to be significantly enhanced 72 h and 7 days post-transfection compared with cells transfected with the miR-control (P<0.05).

Cardiomyocyte apoptosis. Cardiomyocyte apoptosis is critical in the pathology of persistent AF. In the present study, a TUNEL assay demonstrated that the apoptotic rate of cardiomyocytes was significantly decreased following miR-122 inhibition compared with cardiomyocytes in the AF and miR-control groups (P<0.05; Fig. 3).

Expression of apoptosis-associated proteins. The protein expression levels of the pro-apoptotic factor caspase-3 and the anti-apoptotic factor Bcl-x were assessed using western blot analysis. The present results revealed that following miR-122 inhibition in AF cardiomyocytes, the protein expression levels of caspase-3 were significantly decreased compared with untransfected cells (P<0.05; Fig. 4A). Conversely, the protein expression levels of Bcl-x were significantly upregulated following miR-122 inhibition compared with untransfected AF cardiomyocytes and cells transfected with the miR-control (P<0.05; Fig. 4B).

miR-122 inhibition enhances ERK phosphorylation. To further investigate the molecular mechanisms underlying the involvement of miR-122 in AF, the protein expression levels of ERK and p-ERK in cardiomyocytes were assessed using western blot analysis. Following miR-122 inhibition, the p-ERK/ERK ratio was significantly increased in AF cardiomyocytes compared with untransfected AF cardiomyocytes and cells transfected with the miR-control (P<0.05; Fig. 5).



Figure 3. Cardiomyocyte apoptosis was evaluated using the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling assay. Cardiomyocytes were isolated from AF mice and transfected with either a miR-control or miR-122 inhibitor. Untransfected AF cells were used as a control. Representative images are demonstrated in the right panel Data are expressed as the mean \pm standard deviation and the experiments were repeated at least three times. *P<0.05 vs. AF. AF, atrial fibrillation; miR, microRNA.



Figure 4. Protein expression levels of the apoptosis-associated proteins. (A) Caspase-3 and (B) Bcl-x in cardiomyocytes were assessed using western blot analysis. Cardiomyocytes were isolated from AF mice and transfected with either a miR-control or miR-122 inhibitor. Untransfected AF cells were used as a control. Data are expressed as box plots. The experiments were repeated at least three times. *P<0.05 vs. AF. AF, atrial fibrillation; Bcl-x, B-cell lymphoma 2-like 1; miR, microRNA.

Discussion

AF is a major public health concern, as well as a socioeconomic problem, due to its debilitating comorbidities, which include cognitive disturbances, permanent disability and recurrent hospitalization (21). Therefore, the elucidation of the molecular mechanisms underlying AF pathogenesis, and the development of novel therapeutic strategies for the treatment of patients with AF is imperative. The present study demonstrated that the expression of miR-122 was significantly upregulated in cardiomyocytes isolated from mice with AF compared with control mice. Following knockdown of miR-122 expression in AF cardiomyocytes, using transfection with a miR-122 inhibitor, the cell viability of cardiomyocytes was enhanced, whereas their apoptosis was significantly suppressed. Furthermore, the protein expression of the pro-apoptotic factor caspase-3 was significantly decreased, whereas the expression of the anti-apoptotic Bcl-x was significantly upregulated, following miR-122 silencing in AF cardiomyocytes.

miR-122 has been revealed to be highly expressed in liver tissue, where it may be implicated in the regulation of fatty acid metabolism (22,23). Wei *et al* (24) suggested that miR-122 may be associated with the presence and severity of coronary heart disease in patients with hyperlipidemia. Under physiological



Figure 5. Protein expression levels of ERK and p-ERK in cardiomyocytes were assessed using western blot analysis. Cardiomyocytes were isolated from AF mice and transfected with either a miR-control or miR-122 inhibitor. Untransfected AF cells were used as a control. Data are expressed as the mean ± standard deviation and the experiments were repeated at least three times. *P<0.05 vs. AF. ERK, extracellular signal-regulated kinase; p, phosphorylated; miR, microRNA; AF, atrial fibrillation.

conditions, miR-122 expression levels are low in myocardial tissue (12); however, elevated miR-122 plasma levels have been reported in patients with acute heart failure (25). In accordance with the aforementioned studies, the present results revealed that the expression levels of miR-122 were significantly upregulated in cardiomyocytes of mice following the induction of AF compared with control mice. miR-122 has been suggested to be implicated in the maintenance of the differentiated state of tissues, as it may participate in the establishment of tissue-specific gene expression patterns (26). Aberrant expression of miRNAs and the subsequent dysregulation of the expression of their targets have been associated with the proliferation and apoptosis of tumor cells (27). Notably, miR-122 has been demonstrated to regulate the expression of target molecules involved in various biological processes, including cellular proliferation, differentiation and apoptosis (28). The present results revealed that the apoptotic rate of AF cardiomyocytes was significantly decreased following miR-122 silencing compared with untransfected AF cardiomyocytes; similarly, cardiomyocyte viability in the AF group appeared to be lower compared with in the miR-122 inhibitor group. These results suggested that miR-122 may exert pro-apoptotic and anti-proliferative effects on myocardial cells.

Apoptosis is a complex biological process that enables multicellular organisms to discard unwanted cells during their development (29,30). In order to further investigate the involvement of miR-122 in apoptotic pathways, the expression of apoptosis-related proteins was evaluated in cardiomyocytes. The caspase and Bcl-2 protein families have been identified as key mediators involved in apoptotic pathways in various types of cells (31,32). Caspase-3 is a member of the caspase family and has been identified as a pro-apoptotic protein. Ghavami et al (33) revealed that caspase-3 was activated in apoptotic cells via intrinsic and extrinsic pathways. Bcl-x has been identified as an anti-apoptotic protein, and its overexpression has been revealed to inhibit cell death (34). Constitutive activation of the epidermal growth factor receptor has been reported to increase Bcl-x expression, thus suppressing apoptosis (35). In the present study, Bcl-x was demonstrated to be downregulated in cardiomyocytes isolated from mice with AF, thus suggesting the activation of apoptotic pathways. Notably, following miR-122 inhibition, Bcl-x expression was upregulated, whereas caspase-3 expression was downregulated in AF cardiomyocytes. These results suggested that miR-122 may be implicated in the regulation of cardiomyocyte apoptosis in AF, through the modulation of caspase-3 and Bcl-x expression.

ERKs are ubiquitous intracellular signaling molecules, which have been implicated in the regulation of several cellular pathways, including meiosis, mitosis and post-mitotic processes in differentiated cells (36). ERK phosphorylation leads to the activation of kinase activity. Previous studies have suggested that aberrant ERK activation may promote cell death (37,38). Therefore, the protein expression levels of ERK and p-ERK were assessed in cardiomyocytes in the current study, in order to further investigate the molecular mechanisms underlying the effects of miR-122 in myocardial apoptotic pathways. The present results revealed that the p-ERK/ERK ratio in the AF cardiomyocytes was significantly increased following miR-122 silencing, compared with untransfected AF cardiomyocytes, thus suggesting that miR-122 may promote cardiomyocyte apoptosis through the inhibition of ERK activation. Notably, ERK activity has previously been associated with the upregulation of the pro-apoptotic caspase-3 and the downregulation of the anti-apoptotic Bcl-x (37), in accordance with the aforementioned results.

In conclusion, the results of the present study suggested that miR-122 may be associated with the regulation of cardiomyocyte apoptosis in AF. Therefore, miR-122 may have potential as a novel biomarker for the diagnosis of AF, as well as a possible therapeutic target for the development of novel strategies for the treatment of patients with AF.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ made substantial contributions to conception and design and was involved in drafting the manuscript. XZ and WJ performed the experiments. WJ revised the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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