Original Article
miR-let-7e and miR-34a mediate apoptosis induced by ischemic hypoxia of cardiomyocytes in myocardial infarction models

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Abstract: The aim of this present study was to investigate the change of miR-34a and miR-let-7e expression in cardiomyocyte apoptosis induced by hypoxia and ischemia of cardiomyocytes in myocardial infarction models. Real-time PCR was used to quantify miR-34a and miR-let-7e expression in cardiomyocytes under hypoxia incubation and after transfection of mimic-miR-34a, miR-let-7e, and its inhibitor. CCK8 assay and flow cytometry were also performed to detect influence on cell viability, proliferation, and apoptosis after treatment by hypoxia and transfection. RT-PCR analysis showed that expression levels of miR-34a were upregulated, whereas miR-let-7e levels were downregulated in a time-dependent manner in cardiomyocyte and cardium tissues under conditions of ischemic hypoxia. CCK-8 assay and flow cytometry indicated that cell viability could be increased and apoptosis could be inhibited when accompanied by knockout of miR-34a and upregulation of miR-let-7e. Additionally, Western blotting revealed that bcl-2 protein levels were significantly inhibited after knockout of miR-34a of myocardicocytes, whereas they were overexpressed with upregulation of miR-34a (miR-34a-Inhibitor vs NC, p = 0.02; miR-34a-mimic vs NC, p = 0.02). Correlation between circulation expression of miR-34a and miR-let-7e was negative in the myocardium infarction model (Spearman’s correlation analysis, r = -0.942, p<0.001). In conclusion, this study indicates that miR-34a promotes the apoptosis of cardiomyocytes in a myocardial infarction model, whereas miR-let-7e inhibits occurrence of apoptosis. Pro-apoptotic effects of miR-34a may be associated with upregulation of bcl-2 but the mechanisms of miR-let-7e need to be further studied. Therefore, miR-34a and miR-let-7e may be potential therapeutic targets in myocardial infarction.

Keywords: miR-34a, miR-let-7e, apoptosis, cardiomyocytes, myocardial infarction

Introduction
Cardiovascular disease, the leading cause of morbidity and mortality worldwide, is commonly caused by hemodynamic abnormalities in coronary arteries [1]. Global Burden of Disease (GBD) announced that loss from cardiovascular events has reached 300 million years per year, accounting for around 18% of all kinds of diseases [2]. Heart failure and ventricular remodeling have been considered deciding factors in incidence of fatal cardiovascular events, life quality, and prognosis of patients following myocardial infarction. Accumulating evidence has indicated that apoptosis plays a vital role in ventricular remodeling, accelerating the progression of heart failure through remodeling remaining viable cardiomyocytes [3, 4]. Current myocardial infarction (MI) treatment, with a principle of saving the remaining viable myocardium, mainly relies on drug therapy, percutaneous coronary intervention, and surgery therapy [5]. In addition, percutaneous coronary intervention has been considered an important strategy and preferred option to retard or prevent development of heart failure following acute myocardial infarction (AMI) [7]. Many apoptotic cardiomyocytes aggregate in the junction between infarcted and non-infarcted areas after MI. Moreover, after revascularization, a portion of irreversible apoptotic cells aggregate in the myocardial injury area through activation of a series of pathways. Thus, induced by intervention therapy, ischemia-reperfusion injuries have inevitably occurred [6]. Therefore, new therapeutic strate-
gie requires further study to prolong the survival rate of MI patients without ischemia-reperfusion injury. Further study on the mechanisms of myocardial apoptosis, after AMI, could provide new research thoughts on intervention treatment of myocardial apoptosis, thus, providing new ideas for treatment of AMI patients.

MicroRNAs (miRNAs) are a class of small single-stranded noncoding RNAs that interfere with the process of transcription or inhibit translation of target genes. Specific-miRNAs bring the ability of specific gene silencing or suppressing protein expression. Previous reports have suggested that miRNAs are associated with the diagnosis and prognosis of many cancers, including breast, prostate, colon, stomach, and pancreas cancer [8]. Recently, numerous studies have found that miRNAs are also involved in regulation of various physiological and pathophysiological processes in the cardiovascular system, closely related to myocyte aging, hypertrophy, apoptosis, fibrosis, and regeneration [9]. Recently, hypoxia-related miRNAs, considered a necessary target for ventricular remodeling and heart failure, have gradually been recognized. Attractive candidates for prevention of apoptosis, lethal-7 (let-7) and microRNA-34a, highly conservative and specific highly expressed in cardiovascular tissue, have attracted more attention recently. Previous studies have proven that expression of let-7 family members is significantly decreased and miR-34a is evidently upregulated in AMI models [10]. However, the mechanisms between miR-let-7 and miR-34a and apoptosis induced by ischemic hypoxia have not been studied in detail yet.

The purpose of this study was to investigate targets and related pathways of miR-Let-7e and miRNA-34a from the perspective of apoptosis, which has an important significance for the future understanding of development of cardiovascular disease. In addition, roles of miR-Let-7e and miRNA-34a in the treatment of AMI models was also discussed, providing new ideas and strategies for management, treatment, and prognosis of MI patients.

Materials and methods

Cell culture and hypoxia exposure

Cardiomyocytes were isolated, cultured, and passaged according to published literature [11]. Briefly, using small scissors, non-heart tissue was removed from mouse hearts. Heart tissue was washed with fresh Hanks Buffered Saline Solution (HBSS, Gibco, Life Tech, Grand Island, NE, USA). Further cutting of the tissue was then performed until the pieces ranged from 1 to 3 mm. Digestion was performed using gentleMACS technology (Miltenyi, Bergisch Gladbach, Germany), according to manufacturer protocol. Digestion (0.25% Trypsin, Gibco, Life Tech, Grand Island, NE, USA) was ceased and digested tissues were centrifuged for 3 minutes to separate out small non-myocyte cells, such as cardiac endothelial cells and fibroblast when the heart becomes slightly pale and flaccid. Endothelial and immune cells were further separated out using anti-CD144 and anti-CD45 magnetic beads of MACS cell separation (Miltenyi, Bergisch Gladbach Germany), respectively. Afterward, mice cardiomyocytes were inoculated in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Life Tech, Grand Island, NE, USA) and dishes were placed in a 2% CO\textsubscript{2} incubator at 37°C, with subsequent digesting, counting, and re-suspending with 0.25% EDTA-trypsin. The purity of cardiomyocytes was > 95% and cardiomyocytes used in following experiment were in logarithmic growth phase. Mice cardiomyocytes in serum-free DMEM were, respectively, incubated in a humidified chamber with 5% CO\textsubscript{2} and 95% N\textsubscript{2} and a chamber with 5% CO\textsubscript{2} and 95% air. Expression of miR-let-7e and miR-34a was measured by RT-PCR at 0.5 hours, 1 hour, 2 hours, 4 hours and 12 hours.

Acute myocardial infarction model

Acute myocardial infarction models were acquired, following published protocol [12]. Briefly, male, 8- to 10-week-old C57/BL6 mice (Lingchang Biotech, Shanghai, China) were anaesthetized with 2% isoflurane inhalation (Sigma-Aldrich, China). Subsequently, surgical ligation of the left anterior descending coronary artery (LAD) was performed with a prolene suture under mechanical ventilation and analgesia with bupivacaine and carprofen (5 mg/kg subcutaneously, Sigma-Aldrich, China), preoperatively, and every 24 hours for 3 days, postoperatively. After 30 minutes of ischemia, the color of the infarct area changing from red to white was the key to getting successful ligation. Ischemic and hypoxic treated C57/BL6 mice were randomly assigned into four groups (n = 10/group). Expression of miR-let-7e and microR-
NA-34a was measured 6 hours, 24 hours, 72 hours, and 144 hours after LAD ligation.

**Real time PCR analysis**

Expression levels were evaluated by ABI Prism 7700 Sequence Detection System (Applied Biosystems, Life Technologies, Carlsbad, CA), as previously described [13]. RNA was isolated with miRNeasy mini isolation kit, according to manufacturer protocol (Qiagen, Valencia, CA, USA). RNA content and purity were assessed by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). miRNA levels were measured by real-time quantitative (qPCR) analysis (with cDNA used as template for q-PCR) using Fast SYBR Green (Applied Biosystems, Forster City, CA) and an Applied Biosystems Step One Plus machine. Reverse transcription was performed using specific primers for miR-let-7e (mmu-miR-let-7e RT primer: CUAUACGGCCUCCUAGCUUUC) and miR-34a (mmu-miR-34a RT primer: GTGTAATCCAGTGCTGAGTGGAGTTGGCAATTGACTGAGAAGCAACACA). qRT-PCR was performed on Lightcycler 480 (Roche, Switzerland) with SYBR Green III Master and specific primers amplifying miR-34a (F: GGTTGGCAGTGTCTTAGC; R: CAGTGCAGTGAGTGGAGT) and miR-let-7e (F: CGCGCCCCCCGGGCTTATAGTT; R: TATACGGCCTCCTAGCTTTCCCGAGGCTGCGGC). RCR was performed and recycled 50 times as follows: 95°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. Relative expression was quantified with this formula: Relative expression = 2-ΔΔCt; ΔCT = CT-CT reference gene; ΔΔCt = ΔCT-ΔCT control myocardial tissues/cells.

**Cell transfection**

Cardiomyocytes, in the logistic phase, were digested by 0.25% EDTA-trypsin and resuspended to be seeded in 6-well dishes (1×10^5 cells/well). Those dishes were then cultured within DMEM containing 10% FBS, 2 mL-glutamine, penicillin (100 IU/mL), and streptomycin (100 g/mL) overnight. Supernatant was removed and cells were washed by PBS three times. The mimic and inhibitor of miR let-7e (mimic 50 nM, inhibitor 100 nM), miR-34a (mimic 50 Nm, inhibitor 100 nM), and the scrambled sequence (100 nM), regarded as negative control (NC), were transfected to cells in fresh DMEM. After 48 hours, expression of let-7e and miR-34a was quantified by qRT-PCR to evaluate transfection efficiency.

**Cell proliferation assay**

Transfected cardiomyocytes and untreated cardiomyocytes, regarded as control group (1×10/3 mL), were seeded in 96-well plates and cultured in completed medium overnight. Cell viability at day 0, day 1, day 2 and day 4 was quantified by CCK-8 Assay Kit, according to instructions. Briefly, 110 μL mixed medium containing 100 μL fresh DMEM and 10 μL CCK-8 Reagent was added. Plates were then incubated for 2 hours and OD (optical density) value was measured at 450 nm using microplate reader.

**Apoptosis assay**

After 48 hours post-transfection, cells were cultured (around 50% confluency) and hypoxia treated for 2 hours. Apoptosis was then measured by flow cytometry. Untreated cells were used as the control group. Procedures were performed according to instructions. Cardiomyocytes were resuspended and counted, while 1×10^5 cells of 100 μL were stained with PE-Annexin V (5 μL) and 7-AAD (5 μL). Afterward, the mixed solutions were incubated at room temperature for 15 minutes in the dark.

**Western blotting**

Levels of Bcl-2 were measured by Western blotting. Cells (around 50% confluency) were incubated and hypoxia treated for 2 hours. Total protein of those cells treated a different way was extracted using lysis buffer and determined by Bradford assay. SDS-PAGE was then used to separate proteins which were transferred onto nitrocellulose membranes. Moreover, membranes were blocked with 5% milk and incubated with rabbit antibody Bcl-2 (1:20000) and GAPDH (1:1000) at 37°C for 1 hour. After washing, the membranes were co-incubated with anti-rabbit IgG antibody at the same conditions for another 30 minutes. Finally, targeted protein bands were detected with ECL chemiluminescence reagent.

**Assessment of the relationship between miR-34a and miR-let-7e in myocardial infarction model**

After surgical ligation of the left anterior descending coronary artery for 24 hours, MI models (n = 50) were anaesthetized by 10% chloral hydrate (1 mL/100 mg/mice). Blood
samples were collected through cardiac puncture in a centrifuge tube. Serum was then separated by centrifugation at 3000 rpm for 10 minutes. Serological concentrations of miR-34a (n = 25) and miR-let-7e (n = 25) were analyzed using RT-PCR.

Statistical analysis

Continuous variables are presented as mean ± standard deviation (SD). Student’s t-test was used to evaluate differences between the two groups. Mean differences between multiple groups was compared by ANOVA followed by Least Significant Difference (LSD) post hoc test. For histological data, nonparametric Mann-Whitney U test was used. P<0.05 was considered statistically significant.

Results

miR-let-7e and miR-34a expression was influenced through ischemic/hypoxia treatment in vitro and in vivo

Expression levels of miR-let-7e and miR-34a, in vitro and in vivo, were measured by qRT-PCR (Figure 1). The results showed that miR-34a, in cardiomyocyte and cardiac tissues, was obviously upregulated whereas miR-let-7e was evidently attenuated, both in cardiomyocytes and cardiac, under ischemic and hypoxia conditions (144 h: miR-34a 6.34±0.30, p<0.0001; miR-let-7e 0.24±0.05, p<0.0001). This modulation of miR-let-7e was prior to miR-34a in vitro. Relative expression of miR-34a had no significant differences after 6 hours of ischemic treatment (1.06±0.09, p = 0.9943>0.05), but there was obviously decreased in miR-let-7e at the time of 6 hours (0.80±0.07, p = 0.0354<0.05). In vivo, miR-34a and miR-let-7e expression could be distinguished from the control group at 0.5 hours after hypoxia condition (2.28±0.220, p = 0035<0.05; 0.62±0.06, p<0.0001). In addition, the degree of regulation was in a time-dependent manner, both in vitro and in vivo.

miR-34a and miR-let-7e was up-/down-regulated by its mimic and inhibitor

miR-34a and miR-let-7e (mimic or inhibitor) were transfected to cardiomyocytes in the logistic phase to artificially downregulate or upregulate expression of miR-34a and miR-let-7e. Expression levels of miR-34a and miR-let-7e could be increased or decreased by miR-34a and miR-let-7e mimic or inhibitor, as shown in Figure 2. After transfection and culturing for 24 hours, expression levels showed that relative expression compared with control group of mimic-miR-34a and its inhibitor was 4.15±0.11 and 0.32±0.10 (One-way ANOVA, p<0.0001; mimic-miR-34a vs control, p<0.0001; inhibitor-miR-34a vs control, p<0.0001). Additionally, relative expression compared with control group of mimic-miR-let-7e and inhibitor was 4.15±1.32 and 0.32±0.12, respectively (p<0.0001). These results suggest that the process of transfection was effective and the transfected cells could be used for further investigation.
Downregulation of miR34a or upregulation of miR-let-7e enhanced cell viability and inhibited apoptosis of cardiomyocytes

After transfection, cells were incubated in normoxic conditions with complete medium for 48 hours. Additionally, cell viability and proliferation were measured through CCK8 assay at different times. According to Figure 3, no significant differences could be observed in cell viability between those groups the first day after transfection (F = 2.152, p = 0.13>0.05). However, two days later, the results showed that cell viability was significantly increased in miR34a-inhibitor and miR-let-7e-mimic group, while demonstrating lower viability in miR34a-mimic and miR-let-7e-inhibitor group (F = 152.8, p<0.0001). In addition, after transfection and incubation, cells were cultured in hypoxia condition for 2 hours, again, and percentage of apoptosis was recorded by flow cytometry. These results showed that the rate of apoptosis was promoted after transfection with miR-34a-mimic and miR-let-7e-inhibitor, incubated in hypoxia conditions. Additionally, cells after transfection with miR-34a-inhibitor and miR-let-7e-mimic showed much less apoptosis than the control group. However, transfected cells still had a higher percentage of apoptosis when compared with cells incubated under normal oxygen.

miR-34a was positively related to activating of apoptosis through upregulation of Bcl-2

Previous literature has proven that Bcl-2 has the ability of anti-apoptosis. After specifically inhibiting expression of miR-34a with miR-34a-inhibitor and up-regulation of miR-let-7e with miR-let-7e-mimic, cell viability was promoted and rate of apoptosis was also decreased. To further investigate the role of miR-34a and miR-let-7e in the process of apoptosis, expression levels of Bcl-2 were analyzed by Western blotting. As results show in Figure 4A, 4B, expression levels of Bcl-2 protein were reduced accompanied with suppressing expression of miR-34a (0.55±0.15, p = 0.02<0.05). Additionally, expression was significantly increased of Bcl-2 in mimic-miR-34a group (2.15±0.55, p = 0.02<0.05). Moreover, the average folds of mimic-miR-let-7e and inhibitor-miR-let-7e were 1.15±0.34 and 0.88±0.20, whereas there were no significant differences with the control group. Relative relationship between miR-34a and miR-let-7e group was analyzed (Figure 4C), revealing that circulation expression of miR-34a was significantly in inverse proportion to miR-let-7e in a myocardium infarction model (Spearman’s correlation analysis, r = -0.942, p<0.001).
The major findings of this study show that miR-34a regulates levels of Bcl-2 to inhibit viability of and promote the progress of apoptosis of cardiomyocytes. In addition, miR-34a and miR-let-7e are upregulated under the conditions of hypoxia or ischemic treatment. Therefore, miR-34a and miR-let-7e could be therapeutic targets in preventing cardiomyocyte apoptosis of ischemic heart disease.

Regulation of apoptosis is a crucial factor for prognosis after ischemic/hypoxia of cardiomyocytes [14]. Recently, dysregulation of several miRNAs was proven to be closely related to enhanced cell apoptosis and inhibited course of cell proliferation in many diseases [15-17]. miR-34a, an important apoptosis promoter, has been associated with development of cancers including esophageal squamous cell carcinoma by suppressing invasion and metastasis, prostate cancer, colon cancer, hepatocellular carcinoma, and non-small cell lung cancer and lung tumorigenesis [18-22]. It is also involved in pulmonary arterial hypertension through modulating proliferation of smooth muscle cells. Additionally, previous studies have suggested that expression of let-7 family was decreased, of which, miR-let-7e was more significant than any other type in the acute myocardial ischemia model [23]. Recently, many studies have been performed to explore the relationship between let-7 family and cardiovascular disease, considering the feature of high specific expression in cardiovascular disease [24, 25]. The results demonstrated that miR-let-7e could decrease incidence of arrhythmia in acute myocardial ischemia by inhibition of β1-AR, which also participates in the progress of myocardial hypertrophy. However, correlation research between miR-34a and miR-let-7e and apoptosis in myocardial infarction has been rare. Results of this present study show that upregulation of miR-34a and knockdown miR-let-7e can significantly increase the rate of apoptosis in hypoxia-treated cardiomyocytes compared with normal miR-NAs-level group. In contrast, inhibition of miR-34a and overexpression of miR-let-7e can obviously enhance the viability of cardiomyocytes. These results suggest that knockdown of miR-34a and upregulation of miR-let-7e may be potential effective therapeutic methods for ischemic/hypoxic cardiovascular diseases. A crucial role in apoptosis, Bcl-2 blocks most forms of apoptosis by its influence on maintaining mitochondrial membrane integrity and modulating apoptotic signaling pathways. The present study demonstrated that miR-34a promotes expression of Bcl-2. However, miR-let-7e has no statistically significant effect on Bcl-2 expression.

In conclusion, the findings of this study indicate the promotive effects of miR-34a on cardiomyocyte apoptosis, whereas miR-let-7e inhibits...
occurrence of apoptosis. According to results of Western blotting, the pro-apoptotic effects of miR-34a may be associated with upregulation of Bcl-2. The mechanisms of miR-let-7e, however, need to be further studied. Therefore, miR-34a and miR-let-7e may be potential therapeutic approaches in the management of myocardial infarction.

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Disclosure of conflict of interest

None.

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