MiR-34a regulates cell apoptosis after myocardial infarction in rats through the Wnt/β-catenin signaling pathway

J.-H. LI¹, J. DAI¹, B. HAN¹, G.-H. WU², C.-H. WANG³

¹Department of Cardiac Surgery, Beijing Anzhen Hospital, Capital Medical University, Beijing, China
²Beijing Institute of Heart Lung and Blood Vessel Diseases, Beijing Anzhen Hospital, Capital Medical University, Beijing, China
³Department of Cardiology, Beijing Anzhen Hospital, Capital Medical University, Beijing, China

Abstract. – OBJECTIVE: To explore the molecular mechanism of micro ribonucleic acid-34a (miR-34a) in promoting the apoptosis of myocardial cells in the rat model of myocardial infarction (MI).

MATERIALS AND METHODS: Sprague-Dawley (SD) rats were ligated with a left anterior descending branch to construct the MI model. The rats were randomly divided into four groups: sham operation group (Sham group), MI group, MI + miR-34a inhibitor group (MI + miR-34a antagomir group) and MI + miR-34a inhibitor negative control group (MI + antagomir NC group). Echocardiography (ECG) and magnetic resonance imaging (MRI) were adopted to detect the ejection fraction [EF (%)] and fraction shortening [FS (%)] of SD rats. Polymerase chain reaction (PCR) and Western blotting were used to detect expression levels of the apoptotic marker Caspase-3 and genes in Wnt/β-catenin signaling pathway. Hematoxylin and eosin (H&E) staining was applied to detect cardiac injury. In vitro experiments, the rat-derived myocardial cell line H9C2 was selected to simulate myocardial ischemia and hypoxia at the time of MI with an anoxic and serum-free injury model. C59, the Wnt/β-catenin signaling pathway inhibitor was applied in MI + miR-34a antagomir + C59 group, and the effect of miR-34a on the apoptosis of myocardial cells through regulating the Wnt/β-catenin pathway was measured with Real-Time quantitative PCR (qPCR) and 3-(4,5)-dimethylthiazol-(z-y1)-3,5-di-phenyltetrazolium bromide (MTT) cell activity detection kits, respectively.

RESULTS: It was found that after miR-34a antagomir reversed FS (%) and EF (%) in MI rats, the messenger RNA (mRNA) and protein levels of Caspase-3 in Sham group and MI + miR-34a antagomir group were significantly lower than those in the MI group (p < 0.05), indicating that the addition of miR-34a antagomir inhibited myocardial cell apoptosis after infarction, while the mRNA and protein levels of Wnt/β-catenin were both higher than those in the MI group. Besides, H&E staining proved that miR-34a reversed the myocardial injury after MI. Similarly, in vitro experiments showed that, compared with those in Hypoxia group, the level of Caspase-3 decreased in Hypoxia + miR-34a inhibitor group and Sham group, while the apoptosis level in Hypoxia + miR-34a inhibitor + C59 group increased (p < 0.05). The results of the MTT assay were consistent with those of PCR.

CONCLUSIONS: MiR-34a affects myocardial cell apoptosis by regulating the activation and inactivation of the Wnt/β-catenin signaling pathway.

Key Words: Myocardial infarction, MiR-34a, Wnt/D-catenin, Cell apoptosis.

Introduction

Myocardial infarction (MI) is one of the most common cardiovascular diseases in the world. Early and accurate diagnosis is the key to successful treatment and improvement of prognosis. According to epidemiological statistics, MI is becoming more and more common in developing countries. By coronary intervention and postoperative drug maintenance, cardiovascular risk events are reduced, and the long-term survival rate is substantially changed. In the early stage of acute MI, prevention of heart failure and left ventricular remodeling are closely related to the prognosis of patients. Up to now, some circulating biomarkers have been shown to predict cardiovascular events after acute MI, such as N-terminal prohormone of brain natriuretic peptide (NT-ProBNP) and cardiac troponins (cTns). However, early studies reported that the...
The prognostic value of these classical cardiac biomarkers is limited. In recent years, advances in molecular biology and technology have aroused great interests in nucleic acid biomarkers, including non-coding ribonucleic acids (RNAs). These biomarkers may improve the effectiveness of diagnosis or prognosis of MI, which is of great significance to further clarify the mechanism of occurrence and development as well as prevention of the disease.

A microRNA (miRNA) is a non-coding RNA with regulatory function, widely existing in animals, plants, and fungi. It can regulate the gene expression at the post-transcriptional level and participate in life processes, including cell proliferation, apoptosis, and tumors. MiR-34a can be used as an inhibitor of cell proliferation, including tumor cells, and can also participate in regulating the normal function of cells. In addition, the expression of miR-34a in myocardial cells and endothelial cells of patients with cardiac diseases is significantly up-regulated. MiR-34a knockdown can prevent the aging-induced cardiac dysfunction, but whether it is beneficial to the model of myocardial cell proliferation and regeneration after MI remains unclear. This study aims to explore the specific molecular regulation mechanism of miR-34a in promoting apoptosis, inhibiting myocardial regeneration and heart repair and impairing cardiac function in the infarcted heart of the rat MI model. A new prevention strategy for the mechanism of miR-34a in MI is proposed.

Materials and Methods

Materials

H9C2 cell line was purchased from Shanghai Jining Industrial Co., Ltd. (Shanghai, China); Sprague-Dawley (SD) rats from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China); Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) from Gibco (Rockville, MD, USA); Chloroform and isopropanol from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, China); TRIzol from Invitrogen (Carlsbad, CA, USA); Trypsin from Solarbio (Beijing, China); Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA); Messenger ribonucleic acid (mRNA) primers from Shanghai Sangon Biotech Co., Ltd (Shanghai, China); Penicillin-streptomycin from Beyotime (Shanghai, China); Reverse transcription kits from Beijing TransGen Biotech Co., Ltd. (Beijing, China).

MI Animal Model

Male SD rats were randomly divided into sham operation group (Sham group), MI group, MI + miR-34a inhibitor group (MI + miR-34a antagonist group), and MI + miR-34a inhibitor negative control sequence group (MI + antagonist NC group). The left anterior artery was blocked by permanent ligation with a 7-0 propylene suture. Sham group (without ligation of the left anterior descending branch) was the control group. The operation was performed under sterile conditions. Successful establishment of MI model was confirmed by evident S-T segment elevation. At 2 days after the model was established, the rats were intravenously injected with 5 mg/kg miR-34a inhibitors and inhibitor NC, and they were sacrificed at 3 days. Subsequently, the heart tissues were collected for further analysis. This study was approved by the Animal Ethics Committee of Capital Medical University Animal Center.

Cell Culture

The rat embryonic myocardial H9C2 cell line was cultured in a 5% CO₂ incubator at 37°C, and cardiac fibroblasts were cultured in DMEM containing 10% FBS. Cell passage was performed until the cell proliferation reached 70-80%. According to the number of wells paved, the cells were randomly divided into Control group, Hypoxia anoxia model group, Hypoxia + miR-34a inhibitor group, Hypoxia + miR-34a inhibitor +C59 group, and Hypoxia + miR-34a inhibitor + dimethyl sulfoxide (DMSO) group.

Western Blotting Analysis

Tissues were lysed using the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and ground with an electric grinding rod. 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted, and proteins were transferred onto a pure nitrocellulose blotting membrane and blocked with 5% skim milk for 2 h. The Odyssey infrared imaging system (Biosciences, Franklin Lakes, NJ, USA) was applied to detect the immune reactivity, and Image Studio Ver 5.2 software (Lincoln, NE, USA) was used to quantify Western blotting bands.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

1 mL TRIzol reagent was used to extract the total RNA from rat myocardial cells or cardiac
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tissues. The concentration and purity of the extracted RNA were determined using NanoDrop 8000 (Thermo, Waltham, MA, USA). 1 μg RNA was synthesized into single-stranded complementary deoxyribonucleic acids (cDNAs) of miRNAs and long non-coding RNAs (lncRNAs). The relative expression levels of mRNA and miRNA were quantified through miRVana, qRT-PCR and miRNA detection kits and Real-Time RT-PCR using SYBR Green I (Applied Biosystems, Foster City, CA, USA). The relative expression levels of miRNA and mRNA were calculated based on the Ct value, and each sample was standardized with β-actin/U6, respectively. Primer sequences were shown in Table I.

Detection of Cell Proliferation Via 3-[4,5]-Dimethylthiazol-(z-y1)-3,5-Diphenyltetrazoliumbromide (MTT)
The original cell culture medium was discarded, and MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added with 140 μL/well. Cells were incubated at 37°C for 4 h. After the supernatant was discarded, 20 μL DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. Finally, cells were subjected to the EL×80 light absorption enzyme reader (BioTek, Biotek Winooski, Vermont, USA) to record the absorbance value at 490 nm.

Cardiac Function Measurement
After the experimental treatment, SD rats were habituated for 2 months, and their cardiac function (left ventricular function) was tested via MesoMR small animal magnetic resonance imaging (MRI) apparatus and echocardiography (ECG) system (VisualSonics, Toronto, Canada). The MRI solid and liquid nuclear magnetic resonance (NMR) spectrometer Bruker Biospin 800 MHz (Bruker, Billerica, MA, USA) dual-resonance MAS probe was applied to capture high-resolution images. ECG was used to capture high-gradient echoes.

Hematoxylin and Eosin (H&E) Staining
Rat hearts were quickly dissected, immersed in 4% neutral buffered formalin for 7 days, transferred to different concentrations of ethanol for dehydration and embedded in paraffin for histopathological analysis. Thin sections were stained with H&E for histopathological study (Solarbio, Beijing, China).

Statistical Analysis
Data were analyzed by GraphPad Prism 7.0 software (La Jolla, CA, USA) and expressed as mean ± standard deviation. Each experiment was independently conducted for more than three times. The paired t-test was used for the comparison between two groups. p < 0.05 represented that the difference was statistically significant.

Results

Determination of Cardiac Function Indexes in Rats
Sham, MI, MI + miR-34a antagonim, and MI + antagonim NC groups were used as experimental study groups. The results showed that fraction shortening [FS (%)] and ejection fraction [EF (%)] in MI group significantly decreased, which were lower than those in MI + miR-34a antagonim group. The CO values of the four groups of rats did not significantly change and had no statistical significance (p < 0.05) (Table II and Figure 1).

Table I. PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiR-34a</td>
<td>Forward primer 5-CGGTATCATTTTGCTGTCT-3</td>
</tr>
<tr>
<td>Wnt-1</td>
<td>Forward primer 5-TGGTTTGGCAAGGCAACGCA-3</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Forward primer 5-TGATTACAGGCAAACGCAT-3</td>
</tr>
<tr>
<td>U6</td>
<td>Forward primer 5-CGATTTCGACGACAAGGCA-3</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Forward primer 5-CGGTATCATTTTGCTGTCT-3</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward primer 5-GTGCAAGGTCCCAGGAGT-3</td>
</tr>
</tbody>
</table>
Detection of the mRNA Level in Heart Tissue of Rats

As shown in Figure 2, the mRNA expression levels of miR-34a and Caspase-3, as well as the apoptosis rate were notably elevated in MI group, which were reduced in the MI + miR-34a antagomir group. In MI group, Wnt1 and β-catenin were lowly expressed, indicating that the Wnt pathway was inhibited. However, these gene expressions in miR-34a antagomir group increased ($p < 0.05$).

Detection of the Protein Level in the Heart Tissues of Rats

Consistent with PCR results, the protein level of Caspase-3 was markedly increased in MI group but decreased in miR-34a antagomir group. Wnt1, β-catenin were lowly expressed in MI group but highly expressed in miR-34a antagomir group ($p < 0.05$) (Figure 3).

Detection of the mRNA Level in Myocardial Cells

According to Figure 5, the mRNA level of miR-34a was highly expressed in Hypoxia group, but there was no significant difference in its expression between Hypoxia + miR-34a inhibitor group and Hypoxia + miR-34a inhibitor + C59 group. The mRNA expression of Caspase-3 in

Table II. Detection of hemodynamic cardiac function in rats via MRI and ECG ($\bar{x} \pm s$).

<table>
<thead>
<tr>
<th>Group</th>
<th>EDV [μL]</th>
<th>SV [μL]</th>
<th>ESV [μL]</th>
<th>EF (%)</th>
<th>FS (%)</th>
<th>CO [mL/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>79 ± 4.1</td>
<td>53 ± 2.2</td>
<td>24 ± 2.1</td>
<td>68 ± 2.1</td>
<td>48 ± 2.7</td>
<td>27 ± 3.6</td>
</tr>
<tr>
<td>MI</td>
<td>113 ± 4.7</td>
<td>66 ± 2.4</td>
<td>46 ± 1.9</td>
<td>58 ± 2.2*</td>
<td>37 ± 2.6*</td>
<td>29 ± 2.7</td>
</tr>
<tr>
<td>MI+miR-34a antagomir</td>
<td>80 ± 4.2</td>
<td>55 ± 2.1</td>
<td>26 ± 1.8</td>
<td>69 ± 2.4*</td>
<td>46 ± 2.4*</td>
<td>28 ± 2.8</td>
</tr>
<tr>
<td>MI+Antagomir NC</td>
<td>114 ± 4.9</td>
<td>69 ± 2.2</td>
<td>47 ± 2.3</td>
<td>57 ± 1.9</td>
<td>38 ± 2.5</td>
<td>29 ± 3.2</td>
</tr>
</tbody>
</table>

H&E Staining of the Heart Tissues of Rats

H&E staining results manifested that normal myocardial cells in Sham group were orderly arranged, with a clear outline and no fibrosis. However, in MI group, the myocardial cells were disordered in arrangement, blurred in outline, thickened and necrotic in muscle fibers. Fibrous tissue hyperplasia was pronounced. Administration of miR-34a antagomir alleviated cardiac pathology after MI (Figure 4).

Figure 1. Detection of hemodynamic cardiac function indexes, EF, and FS, in rats. *$p < 0.05$. 

Figure 2. Detection of the mRNA level in heart tissue of rats. *$p < 0.05$. 

Figure 3. Detection of the protein level in the heart tissues of rats. *$p < 0.05$. 

Figure 4. H&E staining of the heart tissues of rats. 

Figure 5. Detection of the mRNA level in myocardial cells. 

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Hypoxia group was higher than that in control group, and it was downregulated after the administration of miR-34a inhibitors but increased after C59 inhibited the Wnt signaling pathway. Expressions of Wnt1 and β-catenin decreased in Hypoxia group, recovered significantly after administration of miR-34a inhibitor, and decreased again by C59 treatment \((p < 0.05)\). NS had no evident change (Figure 5).

**Detection of Cell Activity Via MTT**

MTT assay showed that the cell activity was evidently reduced in Hypoxia group but increased Hypoxia + miR-34a inhibitor group increased, indicating that miR-34a inhibitor returns the decreased cell activity triggered by hypoxia (Figure 6). In Hypoxia + miR-34a inhibitor + C59 group, the Wnt signaling pathway was suppressed on the basis of hypoxia injury, resulting in increased apoptosis and decreased cell activity.

**Discussion**

Acute MI has the characteristics of acute onset, rapid change, critical condition, and high mor-
Figure 4. H&E staining of the heart tissues of rats (magnification × 40). In Sham group, the myocardial tissue morphology is normal, and myocardial injury caused by MI is reversed in MI + miR-34a antagomir group.

Figure 5. Detection of the changes in the mRNA expression after hypoxia in myocardial cells via real-time qPCR. *p < 0.05, and NS does not change significantly.
Role of MiR-34a in myocardial infarction

Timely diagnosis and treatment are urgent. With the continuous innovation of basic research and the progress of clinical practice, the treatment plan for MI patients has greatly improved in the past few decades. At present, percutaneous coronary intervention (PCI) is a common treatment. As our understanding of pathophysiology deepens, research on the pathogenesis of MI has shifted. Biotechnology innovations greatly enhance the therapeutic effectiveness, such as gene regulation strategies, affecting many directions such as inflammation, myocardial remodeling, oxidative stress, and angiogenesis. Some studies have shown that the newborn rodent’s heart maintains significant regeneration capacity after heart injury. The process that mediates the regeneration of newborn hearts and the mechanism by which this regeneration ability is lost after birth are still unclear. Consistent with previous reports, this study found that the newborn's heart was less than two years old and almost completely recovered the loss of contractile function caused by MI injury, while the evidence of scar and remodeling was limited.

MiRNA has been identified to be a key regulator in many cell processes including aging, proliferation, and survival. Many researches have revealed that multiple kinds of miRNAs can participate in MI progression. For example, miR-19b-3p, miR-186-5p and miR-134-5p are reported as potential biomarkers for the early diagnosis of MI. The level of miR-34a is differentially expressed in many tissues, including the heart, at different stages. MiR-34a expression in the early stage after birth is closely related to the loss of regeneration potential. Antagonistic expression of miR-34a in adult mouse hearts significantly improves cardiac repair and remodeling after MI. MiR-34a may be a key regulator of cardiac repair/regeneration ability and may be used to enhance endogenous repair of adult hearts after MI. The expression of miR-34a in the infarcted heart and the therapeutic effect of its inhibitors have been previously reported. Studies have confirmed that miR-34a is highly expressed in the heart tissues after MI, and the absence of miR-34a improves cardiac function and reduces apoptosis in the heart. Cell apoptosis is a programmed cell death, and MI-induced hypoxia is an important cause of myocardial cell apoptosis. Considering the loss of myocardial function is one of the core problems of MI, the H9C2 cell hypoxia model was constructed for in vitro verification.

In this study, based on previous experimental results, the specific mechanism of miR-34a in myocardial cell apoptosis after MI was further explored. In vivo and in vitro investigations showed that miR-34a was highly expressed in MI tissues or hypoxia-induced cell models. MiR-34a led to cardiac dysfunction through inhibiting the Wnt/β-catenin pathway, manifesting as reduced cell activity, promoted apoptosis, impaired ability of regeneration. MiR-34a inhibitor suppressed the Wnt/β-catenin signaling pathway, restored the regeneration ability of myocardial cells, and improved the reconstruction of the damaged heart. In view of the anti-cancer effect of miR-34a, the liposome-based miR-34a mimic delivered intravenously has entered phase I clinical trial as an emerging cancer therapeutic agent. The pharmacokinetic and pharmacodynamic properties of the synthesized oligonucleotide optimized by chemical modification have been significantly enhanced, gradually coping with the restriction of the nucleic acid patent medicine. Therefore, in cardiovascular diseases, miR-34a is expected to not only become a clinical diagnostic marker of MI, but also provide a new idea for the treatment of MI.
Conclusions

We found that miR-34a affects myocardial cell apoptosis by regulating the activation and inactivation of the Wnt/β-catenin signaling pathway, and the specific molecular regulation mechanism of miR-34 participating in MI is clarified.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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