Influence of MiR-154 on myocardial apoptosis in rats with acute myocardial infarction through Wnt/β-catenin signaling pathway

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Abstract. OBJECTIVE: To explore the influence of micro ribonucleic acid (miR)-154 on myocardial apoptosis in rats with acute myocardial infarction (AMI), and to analyze whether Wnt/β-catenin signaling pathway was involved in the regulation.

MATERIALS AND METHODS: The Sprague-Dawley (SD) rat model of AMI was established via ligation of left anterior descending artery. Rats were randomly divided into model group (M group, n=12) and ICG-001 intervention group (I group, n=12). At the same time, sham operation group (S group, n=12) was established. In I group, ICG-001 (5 mg/kg) was intraperitoneally injected every day after operation. Meanwhile, an equal amount of normal saline was injected in rats of S group and M group. 21 d after operation, the cardiac function of rats in each group was detected via echocardiography. After that, the rats were immediately executed. MI area in each group was detected via 2,3,5-triphenyltetrazolium chloride (TTC) staining. Myocardial apoptosis level in each group was detected via terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Moreover, the changes of apoptotic proteins in rat myocardial cells were detected via Western blotting. Moreover, the expression level of miR-154 in myocardial cells of rats was detected via quantitative polymerase chain reaction (qPCR). Furthermore, the influence of miR-154 on Wnt/β-catenin signaling pathway was detected via Western blotting.

RESULTS: Compared with S group, left ventricular ejection fraction (LVEF, %) and left ventricular fractional shortening (LVFS, %) were significantly decreased in M group (p<0.01). However, left ventricular internal diameter at end-diastole (LVIDd) and left ventricular internal diameter at end-systole (LVIDs) were significantly increased (p<0.01). In I group, LVEF (%) and LVFS (%) were significantly higher than those of M group (p<0.05), whereas LVIDs and LVIDd were significantly lower (p<0.05). MI area in M group was remarkably larger than that of S group (p<0.01). Meanwhile, MI area in I group was significantly smaller than that of M group (p<0.01). Compared with S group, the number of apoptotic myocardial cells and the protein expression level of cleaved caspase-3 were significantly increased in M group (p<0.01). However, the expression level of B-cell lymphoma-2/Bcl-2 associated X protein (Bcl-2/Bax) was significantly decreased (p<0.01). The number of apoptotic myocardial cells and the protein expression level of cleaved caspase-3 were significantly declined in I group when compared with those of M group (p<0.01). However, the expression level of Bcl-2/Bax was significantly increased in I group (p<0.01). The expression level of miR-154 in myocardial cells of M group and I group was remarkably increased when compared with that of S group (p<0.01). Furthermore, the expression levels of β-catenin and Cyclin D1 in myocardial cells of M group were remarkably higher than those of S group and I group (p<0.01).

CONCLUSIONS: AMI significantly increases the expression level of miR-154. Moreover, miR-154 can activate Wnt/β-catenin signaling pathway, eventually promoting myocardial apoptosis.

Key Words
MiR-154, Myocardial infarction, Wnt/β-catenin signaling pathway, Apoptosis.

Introduction
Acute myocardial infarction (AMI) is an ischemic heart disease, which is manifested as persistent ischemia accompanied by myocardial necrosis or apoptosis. Meanwhile, it is also a common cardiovascular disease seriously threatening human health1-2. The basic pathogenesis of AMI is that the rupture and hemorrhage of coronary atherosclerotic plaques and thrombosis lead to severe luminal stenosis or occlusion. This may result in insufficient or interrupted
myocardial blood supply and myocardial cell structural damage or death, ultimately reducing myocardial contraction and relaxation ability. A large amount of researches have proved that the prognosis of AMI patients is closely related to the area of myocardial necrosis and the number of surviving myocardial cells. Moreover, the non-renewability of myocardial cells indicates that reversing myocardial remodeling and reducing the number of apoptotic myocardial cells are key steps to improve the prognosis of AMI. Currently, angiotensin-converting enzyme inhibitors have been widely applied in the treatment of AMI. However, there is still a lack of specific drugs that can reverse myocardial remodeling in clinic. Furthermore, both morbidity and mortality rates of heart failure after AMI remain high. Wang et al. have found that the expression levels of various micro ribonucleic acids (miRNAs) in myocardial cells of AMI patients are significantly altered. Interference of miRNA expression may become a new means and target for the treatment of AMI. Dong et al. have indicated the abnormal expression of miR-154 in myocardial fibrosis. Inhibition of miR-154 can reduce the phenotypic changes of myocardial fibroblasts and effectively improve myocardial fibrosis. Myocardial apoptosis is an important indicator for poor prognosis of AMI. However, whether miR-154 can regulate myocardial apoptosis through Wnt/β-catenin signaling pathway in myocardial cells has not been elucidated yet. In this experiment, we established a rat model of AMI to study the influence of miR-154 on myocardial apoptosis in AMI. Furthermore, we analyzed whether Wnt/β-catenin signaling pathway was involved in the regulation.

**Materials and Methods**

**Animals and Grouping**

A total of 60 Sprague-Dawley (SD) rats weighing 220-250 g were purchased from the Laboratory Animal Center of Guangzhou University of Chinese Medicine (Laboratory Animal Production License No. SCXK2016-0034, Guangzhou, China). Rats were fed in a specific pathogen-free (SPF) room with humidity of (45±2)% at (23±1)°C under normal day-night rhythm. Meanwhile, all rats had free access to food and water to adapt to the environment for 1 week. The Sprague-Dawley (SD) rat model of AMI was established via ligation of left anterior descending artery. Totally, 24 successfully modeled rats were selected and randomly divided into model group (M group, n=12) and ICG-001 intervention group (I group, n=12), respectively. Normal Sprague-Dawley (SD) rats did not undergo ligation of left anterior descending artery, and the remaining operations were the same as M group. Next, 12 surviving rats were selected in sham operation group (S group). ICG-001 (5 mg/kg) was intraperitoneally injected as an intervention in I group every day after operation. However, an equal amount of normal saline was injected in S group and M group. ICG-001 is a kind of specific inhibitor of Wnt/β-catenin signaling pathway, which can effectively inhibit the production of β-catenin and block Wnt/β-catenin signaling pathway. All experiments were in line with the guidelines for feeding and use of laboratory animals issued by the National Institutes of Health. The animal experimental scheme was reviewed and approved by the Ethics Committee of Xinxiang Medical University Animal Center (Weihui, China).

**Establishment of AMI Model**

AMI model was established via ligation of left anterior descending artery. Rats were first anesthetized with 10% chloral hydrate and fixed on an operating table. After tracheal intubation, small animal ventilator was connected to assist respiration. The parameters of ventilator were adjusted as follows: tidal volume: 5 mL, respiratory rate: 80 beats/min, and respiratory ratio: 5:4. The chest hair was removed and the chest was cut open between the 3rd and 4th intercostal spaces to expose the heart. The left anterior descending artery was ligated at about 2 mm on the lower edge of left auricle using the 6-0 wire. Whitening of cardiac apex indicated the successful operation. After the chest was sutured and closed, the rats were treated with anti-infective therapy using penicillin. After resuscitation, the trachea cannula was removed and the rats were placed into the insulation cage.

**Determination of Cardiac Function**

The cardiac function of rats in each group was detected using Vevo high-resolution small animal ultrasonic imaging system at 21 d after AMI. The rats were first anesthetized with 2% isoflurane and fixed on a thermostatic operating table. The limbs were connected to electrocardiogram electrodes. After the chest hair was removed, the ultrasonic sound-conducting agent was applied on the chest for ultrasonic detection. Left ventricular internal diameter at end-systole (LVIDs), left ventricular internal diameter at end-diastole (LVIDd), left
ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) of rats were recorded to evaluate cardiac function.

**Determination of MI Area**

The rats were sacrificed immediately after the determination of cardiac function. The heart was taken and trimmed. Meanwhile, the left ventricle was collected, weighed and sliced into sections. After incubation with pre-heated 1% TTC solution (Beijing Dingguo Biotechnology Co., Ltd., Beijing, China) at 37°C for 15 min in dark, the sections were fixed with 4% paraformaldehyde for 30 min and photographed. The staining area was measured using Image-Pro Plus image analysis software. MI area was calculated by the following formula: MI area (%) = (MI area per section/myocardial area in the section * myocardial weight in the section) ∑/left ventricular weight * 100%.

**Detection of Myocardial Apoptosis Level**

The rats were executed immediately after the determination of cardiac function. Ischemic tissues of left ventricle were taken and prepared into paraffin sections. The level of myocardial apoptosis in each group was detected according to the instructions of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). The number of apoptotic myocardial cells in each group was observed under a confocal fluorescence microscope (Nikon, Tokyo, Japan). After photography, the number of TUNEL-positive cells (green fluorescence) in myocardial tissues of each group was calculated.

**Detection of Protein Expression Levels via Western blotting**

The rats were executed immediately after the determination of cardiac function. Ischemic tissues of left ventricle were taken, weighed and lysed using RIPA lysis buffer (100 mg/mL) (Beiyotime Biotechnology, Shanghai, China). After homogenization and centrifugation, the supernatant was collected as total protein sample. The concentration of extracted protein in each group was detected using the bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA). Subsequently, an equal concentration of protein sample was prepared and inactivated via water bath for 15 min. Extracted protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (IPVH00010, Millipore, Billerica, MA, USA). After sealing with 5% skim milk powder for 2 h, the membranes were incubated with primary antibodies of cleaved caspase-3, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), β-catenin, Cyclin D1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (purchased from CST, diluted at 1:1000, Danvers, MA, USA) at 4°C overnight. After washing with Tris-buffered saline and Tween 20 (TBST) for 3 times, the membranes were incubated again with secondary antibodies (Beyotime Biotechnology, Shanghai, China) at room temperature for 1 h. Then, the membranes were washed again with TBST for 3 times. Immunoreactive bands were developed and analyzed using electrochemiluminescence (ECL) solution on a developing machine (Thermo Fisher Scientific, Waltham, MA, USA).

**Detection of miR-154 Level in Myocardial Cells via Quantitative Polymerase Chain Reaction (qPCR)**

The rats were executed immediately after the determination of cardiac function. Ischemic tissues of left ventricle were taken, and total RNA was extracted from hippocampus in each group using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Optical density (OD) value was then determined. Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using reverse transcription kit (TaKaRa, Otsu, Shiga, Japan) and stored in a refrigerator at -20°C. Specific reaction conditions were as follows: 42°C for 15 min and 95°C for 2 min. The qPCR system was prepared, and the reaction conditions were as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, for a total of 30 cycles, and extension at 72°C for 5 min. The relative expression level of gene was calculated by the 2-ΔΔCt method. The primers were synthesized by Invitrogen (Carlsbad, CA, USA), and the sequences were shown in Table I (GAPDH as an internal reference).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences of PCR primers</th>
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<tr>
<td>MiR-154</td>
<td>Sense ACACCTCCAGCTGGGTAG</td>
</tr>
<tr>
<td></td>
<td>Antisense CTCAACTGGTGTCGTGGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense TATGGACGGCTGTTAC</td>
</tr>
<tr>
<td></td>
<td>Antisense TTCCCATTCTCACGCCTTG</td>
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Table I. Primer sequences.
**Statistical Analysis**

Experimental data were expressed as (x±s). GraphPad Prism 6.0 (La Jolla, CA, USA) was used for one-way analysis of variance. Tukey’s test was adopted for pairwise comparison of multiple samples. Least Significant Difference (LSD) t-test was adopted for data comparison between two groups. p<0.05 was considered statistically significant.

**Results**

**Cardiac Function**

The cardiac function of rats in each group was detected using high-resolution small animal ultrasonic imaging system at 21 d after AMI. As shown in Figure 1, LVEF (%) and LVFS (%) were significantly decreased in M group when compared with S group (p<0.01). However, LVIDd and LVIDs were significantly increased in M group (p<0.01). In I group, LVEF (%) and LVFS (%) were significantly higher than those of M group (p<0.05), whereas LVIDs and LVIDd were remarkably lower (p<0.05).

**MI Area**

The heart was collected from rats in each group at 21 d after AMI. The MI area in each group was detected via TTC staining. It was found that the left ventricular MI area of M group was significantly larger than that of S group (p<0.01). However, MI area of in I group was significantly smaller than that of M group after ICG-001 intervention (p<0.01) (Figure 2).

**Myocardial Apoptosis Level**

The heart was collected from rats in each group at 21 d after AMI. The level of myocardial apoptosis in each group was detected via TUNEL staining. As shown in Figure 3, a large number of TUNEL-positive cells were observed in myocardial cells of M group. The number of TUNEL-positive cells in M group was significantly larger than that of S group (p<0.01). Furthermore, the number of TUNEL-positive cells was significantly smaller in I group than M group (p<0.01).

![Figure 1. Cardiac function. A, LVEF: left ventricular ejection fraction, B, LVFS: left ventricular fractional shortening, C, LVIDd: left ventricular internal diameter at end-diastole and D, LVIDs: left ventricular internal diameter at end-systole. **p<0.01 vs. S group, *p<0.05 vs. M group.](image)
Expression Levels of Apoptosis-Related Proteins

The heart was collected from rats in each group at 21 d after AMI. The expression levels of apoptosis-related proteins in myocardial cells of each group were detected via Western blotting. Compared with S group, the protein expression level of cleaved caspase-3 was significantly increased in M group ($p<0.01$), whereas Bcl-2/Bax expression level was remarkably decreased ($p<0.01$). Meanwhile, the protein expression level of cleaved caspase-3 significantly declined in I group when compared with M group ($p<0.01$). However, the protein expression of Bcl-2/Bax was significantly increased in I group ($p<0.01$) (Figure 4).
Expression Level of miR-154

The heart was collected from rats in each group at 21 d after AMI. The expression of miR-154 in myocardial cells of each group was detected via qPCR. The results showed that the mRNA expression level of miR-154 in myocardial cells of M group and I group was remarkably increased when compared with that of S group (p<0.01) (Figure 5).

Influence of miR-154 on Wnt/β-Catenin Signaling Pathway

The heart was collected from rats in each group at 21 d after AMI. The protein expression levels of β-catenin and Cyclin D1 in myocardial cells of each group were detected via Western blotting. The results revealed that the protein expression levels of β-catenin and Cyclin D1 in myocardial cells of M group were remarkably higher than those of S group (p<0.01). However, they were significantly declined in I group after ICG-100 intervention (p<0.01) (Figure 6).

Discussion

Apoptosis is a form of cell death in the body under pathological or physiological conditions. Apoptotic cells will be phagocytized by macrophages without causing damage to adjacent normal cells. Therefore, it plays an extremely important role in the growth and development of the body, as well as maintenance of homeostasis. Previous studies have indicated that AMI or myocardial ischemia-reperfusion can lead to myocardial apoptosis. Meanwhile, the number of apoptotic cells in ischemic regions is significant.

Figure 4. Changes in apoptosis-related proteins detected via Western blotting. A. Protein band, B. Statistical graph of cleaved caspase-3 and C. Statistical graph of Bcl-2/Bax. **p<0.01 vs. S group, *p<0.01 vs. M group.

Figure 5. MiR-154 expression level detected via qPCR. "p<0.01 vs. S group, **p<0.01 vs. M group.
ly larger than that of non-ischemic regions. The apoptosis of a large number of myocardial cells will result in ventricular remodeling and heart failure\(^\text{13}\). Cell apoptosis includes: Caspase-dependent apoptosis and non-Caspase-dependent apoptosis. Caspase, as a kind of cysteine protease, can regulate the transduction of various signals in the body. Caspase3, as an apoptotic executioner, leads to apoptosis through cleaving protein substrates\(^\text{14}\). Bcl-2 inhibits apoptosis, whereas Bax promotes apoptosis. Furthermore, Bel-2/Bax regulates apoptosis in the body\(^\text{15}\). Research has suggested that miRNA regulates the apoptotic process in vivo. For example, miR-133 inhibits apoptosis and protects cells through inhibiting caspase9 expression in cells\(^\text{16}\). Zhang et al\(^\text{17}\) have found that the expression level of miR-199a is significantly declined in myocardial ischemia. Meanwhile, apoptosis is promoted by regulating HIF-α. In this study, it was found that the apoptotic level of myocardial cells in AMI rats was significantly increased. The expression of cleaved caspase-3 was also significantly increased. However, Bcl-2/Bax expression level was significantly decreased. Our findings also indicated that the expression level of miR-154 was significantly increased in myocardial cells of M group. After ICG-100 treatment, the expression of cleaved caspase-3 was significantly decreased, whereas Bcl-2/Bax was increased.

Moreover, it had no significant influence on the expression of miR-154. Sun et al\(^\text{18}\) have found that miR-154 can activate Wnt/β-catenin signaling pathway through targeting DKK2, thus affecting myocardial fibrosis. In this study, the expressions of β-catenin and Cyclin D1 in myocardial cells of AMI rats were remarkably increased. This indicated that AMI could increase the expression of miR-154 and further activate Wnt/β-catenin signaling pathway, thereby mediating cell apoptosis. After treatment with ICG-100, a specific inhibitor of Wnt/β-catenin signaling pathway, the expressions of β-catenin and Cyclin D1 were significantly reduced. However, ICG-10 had no significant influence on the expression of miR-154. These results suggested that miR-154 could activate Wnt/β-catenin signaling pathway to promote myocardial apoptosis. Moreover, inhibiting β-catenin could effectively reduce AMI-induced myocardial apoptosis. ICG-100 has been proved to specifically inhibit the expression of β-catenin, thereby blocking Wnt/β-catenin signaling pathway. Therefore, it is often used as a tool for studying the physiological functions of Wnt/β-catenin signaling pathway\(^\text{19}\). Dong et al\(^\text{9}\) have demonstrated that miR-154 knockout can significantly reduce myocardial fibrosis and apoptosis. The underlying mechanism may be the inhibition of Wnt/β-catenin signaling pathway.
Role of MiR-154 in rats with acute myocardial infarction

Conclusions

We found that AMI significantly increased the expression level of miR-154. Meanwhile, miR-154 could promote myocardial apoptosis through activating Wnt/β-catenin signaling pathway. In addition, inhibiting β-catenin could significantly reduce AMI-induced MI and myocardial apoptosis.

Conflict of Interests

The authors declared no conflict of interest.

References