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MiR-101a attenuates myocardial cell apoptosis in rats with acute myocardial infarction via targeting TGF-β/JNK signaling pathway

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Abstract. – OBJECTIVE: To investigate the effect of micro ribonucleic acid (miR)-101a on myocardial cell apoptosis in the rat model of acute myocardial infarction (AMI) and its regulatory mechanism.

MATERIALS AND METHODS: A total of 30 Sprague-Dawley (SD) rats were randomly divided into the Sham group, Model group, and miR-101a mimic group, with 10 rats in each group. The rat model of AMI was established by the ligation of the anterior descending coronary artery. The rat left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) were detected using a color Doppler ultrasonic apparatus. Subsequently, the online database (TargetScan) was adopted to predict miRNAs that could be also regulate TGF-β1. Hematoxylin and eosin (HE) staining was conducted to reveal the histopathological morphology changes in the rat heart. The serum levels of cysteinyl aspartate specific proteinase-3 (Caspase-3) and Bcl-2-associated X protein (Bax) in rats were detected via enzyme-linked immunosorbent assay (ELISA). Moreover, the expression levels of the transforming growth factor-β1 (TGF-β1) and c-Jun N-terminal kinase (JNK) in rat heart were measured via Western blotting.

RESULTS: Through searching miRNA database, miR-101a and TGF-β1 messenger RNAs (mRNAs) had binding sites in the 3' untranslated region (3' UTR). Compared with those in Sham group, the rat LVEDV and LVESV were notably elevated, the histopathological morphology of the heart was obviously damaged, the apoptotic rate of myocardial cells and the levels of TGF-β1 and JNK proteins significantly increased in the Model group. Additionally, compared with those in the Model group, the LVEDV and LVESV of rats in miR-101a mimic group were significantly reduced, the histopathological morphology of the heart was markedly improved, and the apoptotic rate and the levels of TGF-β1 and JNK in rat heart were remarkably decreased.

CONCLUSIONS: The myocardial cell apoptosis in AMI rats can be suppressed by overexpression of miR-101a by inhibiting the TGF-β1/JNK signaling pathway.

Key Words:
miR-101a, TGF-β1/JNK signaling pathway, Acute myocardial infarction, Myocardial cell, Apoptosis.

Introduction

Acute myocardial infarction (AMI) is a primary cause of death in human beings. Its incidence rate has increased year by year because of the poor life quality, eating habit changes, and environmental factors[1,2]. The number of patients with cardiovascular diseases reached 290 million in China according to the cardiovascular disease report published in 2017. Early diagnosis and timely treatment of AMI can effectively reduce its incidence rate, alleviating the burden on the families and society. The clinical manifestations, such as arrhythmia, cardiac remodeling, and heart failure, occur in patients with AMI. Due to an extremely high mortality rate, AMI has attracted much attention[3]. The pathogenesis of AMI is complex and remains unclear yet. In recent years, it has been found that the long-term acute and persistent coronary ischemia finally lead to the apoptosis and the loss of a large number of myocardial cells[4]. The developing drugs that can inhibit the myocardial cell apoptosis will bring new hope for the treatment

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of AMI. Besides, a search for effective molecular targets is urgently needed.

The transforming growth factor-beta l (TGF-β1) is a cytokine that can regulate cell proliferation, differentiation, migration, and other biological processes[5]. It is a polypeptide formed by the combination of two single chains with the molecular weight of 11 kD through disulfide bonds. The stimulation of TGF-β1 activates the downstream mitogen-activated protein kinase (MAPK) pathway. The c-Jun N-terminal kinase (JNK), a member of the third major MAPK family, is a crucial downstream target of TGF-β1. JNK participates in regulating the biological process of cell apoptosis. It is reported that the activated TGF-β1 under external stimuli thereafter stimulates the activation of JNK to promote cell apoptosis[6]. The above findings, therefore, suggested that inactivation of TGF-β1/JNK signaling pathway can effectively suppress the apoptosis of myocardial cells.

A micro ribonucleic acid (miRNA) belongs to a branch of genomics and is currently a hot topic in the field of life science. It is a non-coding, single-stranded, small molecule with 19-25 nucleotides in length. By binding to the 3'untran d region (3'UTR) of messenger RNAs (m miRNAs inhibit the translation of mRNAs o- mote their degradation, so as to regulate their scription levels[7,8]. In recent years, miRNAs h become a hot topic in the dise gression Some researchers have foun bnorma expressions of miRNAs ar sely re d to tumors, neurodegenerative ses, o etc. According to rece st [10] an indispensable ro the f of the heart, lung, liver, and o organs. So dies have manifested tha R-101a expr is remarkably do egul a MI rats. Hence, this study inte to explore rotective effect of miR-101 the heart tissue AMI rats and to invest its m hanism.

terial d Methods

Re nts

-β1, JNK, and β-actin primary antibodies w from Wanlei Biotechnology Co., . (Shenyang, China), the horseradish peroxi e (HRP)-labeled secondary antibodies from Danvers, MA, USA). The hematoxylin and eos (H&E) staining solution was purchased from Beijing Solarbio Life Science Co., Ltd. (Beijing, China), the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit from R&D Systems (Minneapolis, MN, USA), and miR-101a mimics were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, Chin

Instruments

The ultra-low temperature ator was bought from Eppendorf (H mburg many), electrophoresis apparatus a embran fer apparatus from Bio-Ra ercules, CA, the Sequoia 512 color ppler sound d nostic instrument fr EM (Berli Germany), and the m osc Niko okyo, Japan).

Animals

The s ecific gen free (SPF) male Sprague Dawley (SD weighing 230±10 g we ased from aboratory Animal C r of Guizhou Medical University. They were sed under th oom temperature of 22±1°C humidity of 2% and had free accesses to w and food s study was approved by the An ommittee of Gaotang County People s tal of Shandong Animal Center.

ation of the Rat Model of AMI

ats were intraperitoneally injected with 10% chloral hydrate for anesthesia and fixed on the operating table in the supine position. The operating area was disinfected. After that, the skin was cut between the 4th and the 5th rib on the left side of the chest, and the muscle was peeled off with a blunt separator to expose the heart. The pericardium was opened under a microscope and ligated at the junction of the pulmonary artery cone and left ventricle. After ligation, the myocardium turned white. The incision was sutured layer by layer, followed by disinfection and injection with penicillin for preventing infection. Finally, it was observed in electrocardiogram that the ST segment was continuously elevated, with Q wave and ventricular arrhythmia, indicating the successful preparation of the MI model.

MiRNA Online Database Analysis of the Targeted Binding of MiR-101a to TGF-Ð1

TargetScan database is a website for miRNA target gene prediction, including miRNA target gene results of human, mouse, fruit fly, nematode, zebrafish, and other species and contains conservative and non-conservative sequences. We used TargetScan database (http://www.targetscan.org/) and defined 'TGF-β1' as the key word. The po-

tential binding sites with reference to the pop-up page were searched.

Detection of Pathological Changes in Heart Tissues of Rats via H&E Staining

The rat heart tissues were fixed with 4% paraformaldehyde and dehydrated with 80% methanol solution for tissue embedding. Then the tissues were cut into slices with a thickness of 8 μm. After that, the slices were immersed in xylene solution, dewaxed, and dehydrated with 100%, 90%, 80% and 70% ethanol solution. After rinsing with running water for 5 min, the slices were stained by hematoxylin for 3 min, rinsed with running water for 5 min, counter-stained by eosin solution for 3 min, rinsed with ethanol solution at the low-to-high concentrations, and finally sealed in neutral resin.

Detection of Apoptosis of Heart Tissue Cells of Rats via TUNEL Assay

The rat heart tissues were cut into slices, the air dried at room temperature, and fixed with 4% paraformaldehyde. After immersing in cell membrane permeable solution for 20 min, the slices were incubated with 1 mL of protease K working solution for 15 min. After that, they were incubated with 100 μL of buffer solution for 1 h, washed with phosphate-buffered saline (PBS), and incubated with staining solution. Finally, 4',6-diamidino-2-phenylindole (DAPI) staining (Sigma-Aldrich, St. Louis, MO, USA) was performed before image capture under a microscope.

Detection of the Expressions of TGF-β1 and JNK Proteins in Heart Tissues of Rats via Western Blotting

50 mg tissues were lysed and centrifuged at 12000 rpm for 10 min, and the supernatant was retained. After rising, the samples were subjected to electrophoresis with 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) using the wet method and sealed in bovine serum albumin (BSA). After washing with Tris-Buffered Saline and Tween-20 (TBST) solution, the membranes were incubated with primary antibodies (TGF-β1, 1:1000, and JNK, 1:1000) overnight, and secondary antibodies for 1 h. Ultimately, the band exposure was performed with diaminobenzidine (DAB) solution (Solarbio, Beijing, China), and the optical density of the bands was analyzed with ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

The data of each group were expressed by mean ± standard deviation, data were analyzed and plotted using GraphPad 5 software (La Jolla, CA, USA). The comparison between the groups was using one-way ANOVA test, followed by the post hoc test (Least Significant Difference). $p<0.05$ was considered that the difference was significant.

Results

Successful Preparation of the AMI Model in Rats

After the preparation of the AMI model in rats, left ventricular end-systolic volume (LVESV) and left ventricular end-diastolic volume (LVEDV) levels were measured by small animal echocardiography. The results revealed that the LVESV and LVEDV levels in Model group were markedly elevated compared with those in the Sham group ($^{*}p<0.05$, $^{*}p<0.05$), while those in miR-101a mimic group were significantly reduced, compared with those in the Model group ($^{\#}p<0.05$, $^{\#}p<0.05$) (Table I).

TGF-β1: a Target Downstream Gene of MiR-101a

By searching in the TargetScan database, the pop-up page was shown in Figure 1A. Then miR-101a was clicked to view the binding sites to the 3'UTR of the TGF-β1 mRNA (Figure 1B), proving that there were potential binding sites between miR-101a and TGF-β1.

Table I. LVESV and LVEDV levels in the heart of rats.

Group	Sham	Model	MiR-101a mimic
LVESV (μL)	67.36±8.56	298.47±13.28*	165.72±9.37#
LVEDV (μL)	320.90±12.46	503.63±12.83*	429.46±8.65#

Note: *$p<0.05$: Model group vs. Sham group, and $^{\#}p<0.05$: miR-101a mimic group *vs.* Model group.

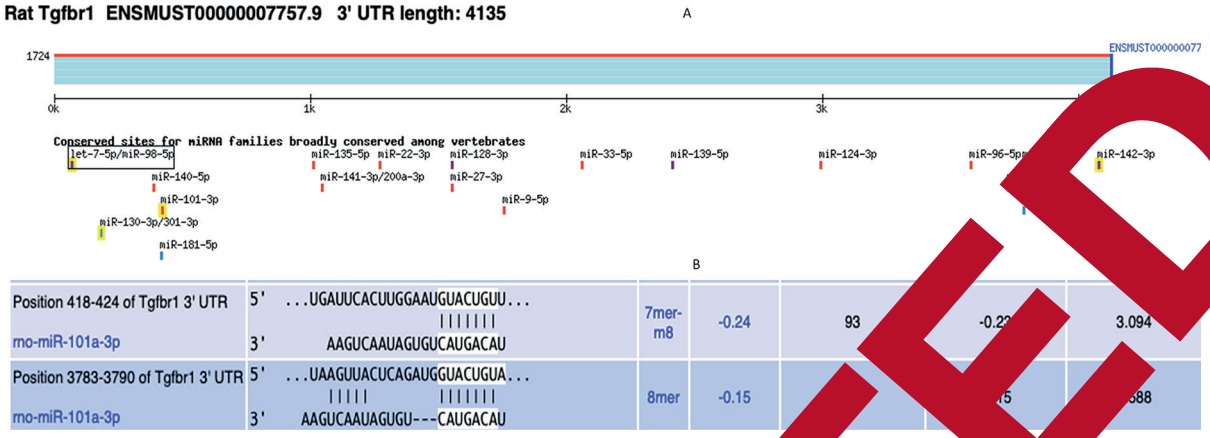

Figure 1. TGF-β1: a target downstream gene of miR-101a. ***A,*** TargetScan datab is of miRNA regulate the TGF-β1 expression. ***B,*** Binding site between miR-101a and TGF-β1.

MiR-101a Mimics Could Improve the Cardiac Histopathological Morphology of AMI Model Rats

The myocardial cells in the Sham group were arranged orderly, compact, and clear in structure. However, those in the Model group re barely alive, with a larger volume, a dis arrangement, and a larger intercellular spac f- ter treatment with miR-101a mimics, the ca histopathological morphology of AMI rats v evidently improved (Figure 2).

MiR-101a Mimics Coul duce Apoptosis Rate of He Tissue AMI Model Rats

According to TU L sta esults, compared with that in Sham grou apoptotic rate of myocar in the Mod up sig- ntly increased ($^{*}p<0.05$) (Figure 3A). Com- d with that i e Model group, the apoptotic of myocardi lls in miR-101a mimic group decreas ($^{\#}p<0.05$) (Figure 3B). The abo ated that miR-101a overexpression ca ess the apoptosis of myocardial lls in AMI rats.

a Overexpression Could Inhibit the Protein Levels of TGF-β1 and JNK

Western blotting band results manifested that compared with those in the Sham group, the expressions of TGF-β1 and JNK in the heart tissues of rats in the Model group were significantly upregulated ($^{*}p<0.05$) (Figure 4A). TGF-β1 and JNK levels in miR-101a mimic group were markedly declined compared with those in the Model group ($^{\#}p<0.05$) (Figure 4).

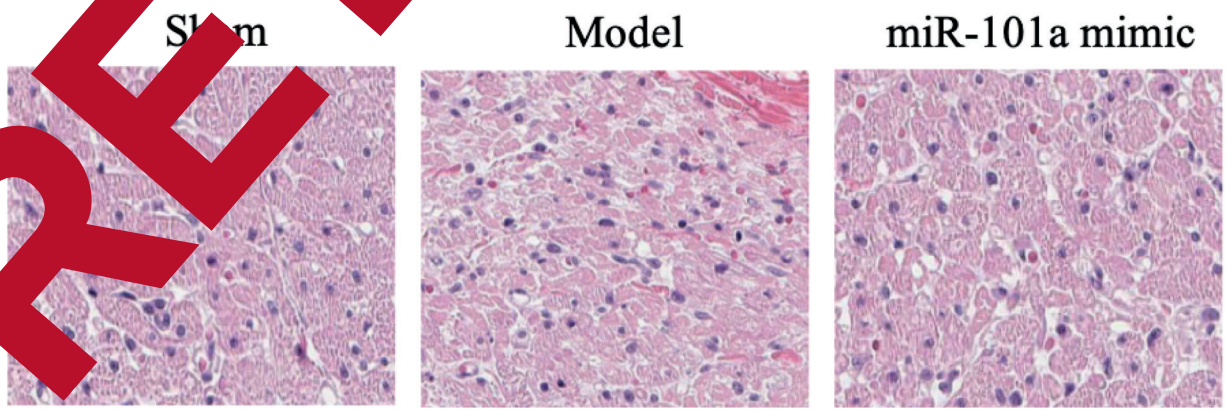

Figure 2. Histopathological changes in the heart of rats (magnification 40×).

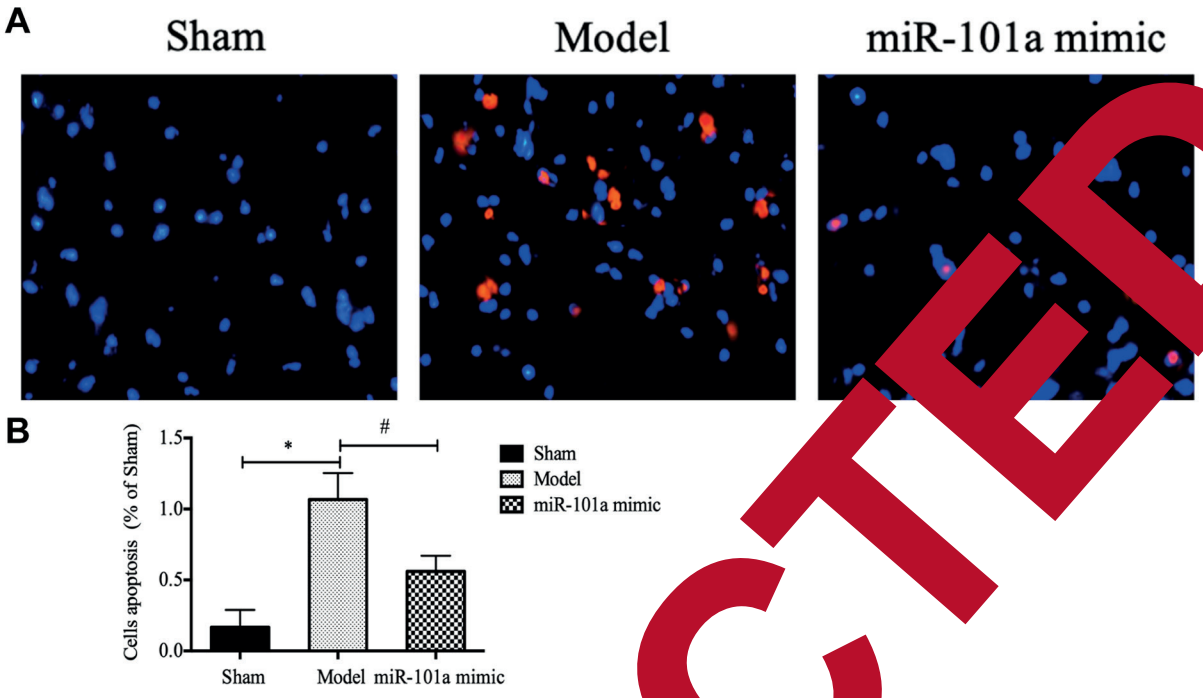

Figure 3. Apoptosis of myocardial cells in rats detected via TUNEL staining. ***A***, TUNEL staining results (magnification 20×). ***B***, Apoptosis rate ($^{*}p<0.05$, $^{\#}p<0.05$).

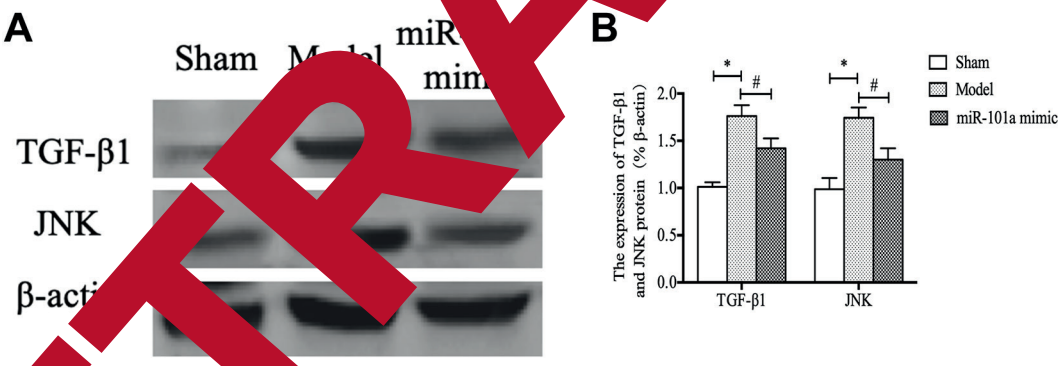

Figure 4. Expressions of TGF-β1 and JNK proteins detected via Western blotting. ***A***, Western blotting bands. ***B***, Statistical graph of Western blotting bands ($^{*}p<0.05$, $^{\#}p<0.05$).

Discussion

About 500,000 people die of AMI each year in China according to incomplete statistics, and the data showed an evident upward trend in recent years, which has brought a serious economic burden to families and the society[11]. Currently, AMI is mainly treated with surgery, intervention, drugs, and other methods. However, these therapeutic approaches can only delay the development of the disease and cannot radically cure it[12,13]. AMI is the result of many causes, among which the theory of abnormal apoptosis of myocardial cells is generally accepted[14]. Liu et al[15] found that the silence of cysteinyl aspartate specific proteinase-3 (Caspase-3) could alleviate myocardial cell apoptosis and narrow down MI area, indicating that the inhibition of cardiomyocyte apoptosis can notably improve the cardiac function of MI rats. Tao et al[16] found that a strengthening in the exer-

cise training can reduce the MI area of AMI rats and eliminate MI-induced autophagy and apoptosis. The above researches suggested that the effective inhibition of myocardial cell apoptosis will bring a new therapeutic scheme for the treatment of AMI.

MiRNAs are the key molecules of medical research in recent years. They participate in the regulation of cell growth, development, differentiation, and aging in the form of networks[17]. MiRNAs are widely expressed in organisms and have tissue specificity. MiRNAs can be specifically expressed in different tissues, organs, blood, and cerebrospinal fluid. Abnormal manifestations of miRNAs in the body can lead to the occurrence or development of diseases. Zhu et al[18] found a significant difference in miR-133a expression through the comparative analysis of the data of patients with AMI and normal people in PubMed, EMBASE, and Cochrane libraries. It is indicated that miR-133a can be used as a diagnostic biomarker for AMI. Another meta-analysis study on AMI showed that miR-208b is evidently up-regulated in the serum of AMI patients, indicating that miR-208b can be used as a biomarker for ea... agnosis of AMI[19]. MiR-101a has been ext... studied in tumors and nephropathy, and mi...a has been confirmed to be a biomarker for tu... and nephropathy, but its correlation with AMI ... been rarely reported. Ding et al[20] ...vered th... miR-101a can regulate the TG... signaling pathway, so as to prote...inst h...tensive nephropathy. It has been ...ied in ... ber of studies that miR...A... ... the occurrence and d...lopme... MI.

In this study, t...ore, the lig... of the anterior descendi... ...ary artery ...adopted to prepare th... mo... AMI, and the targeted bindin... miR-101a t... ...F-β1 was analyzed using th...RNAs online ...se. The results prove... exist...ce of potential binding targets bet... them... ...bsequently, H&E staining was con... ...ect the ...hological morphology. It was ...hat mi...1a overexpression could ...kably ...ro...e damage to the cardiac his...tholog...orphology and significantly red... the LVE... and LVEDV levels, suggesti... ...01a overexpression can evidently ...ance the cardiac function of AMI rats. Final... TUNEL results demonstrated that miR-101a ...pression significantly suppressed myocardia... apoptosis. In order to study its regulatory mechanism, two important targets on the TGF-β1/JNK signaling pathway were detected by Western blotting. According to the results, miR-101a overexpression markedly downregulated the expression levels of TGF-β1 and JNK. The above results indicated that miR-101a over... can suppress the apoptosis of myoca... cells ... AMI rats, by inhibiting TGF-β1/... signaling pathway. The experimental resu... ...ide a new basis and strategy for the applicatio... ...R-101a in the treatment of AMI.

Co... ...ion

We found t... ...myocar... ...ell ...tosis in AMI rats ca... suppressed ... ...verexpression of mi... ...d its mecha... may be associated ... the ... ...tion of the TGF-β1/JNK signaling pathway.

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...uthors declare ...onflicts of interest.

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