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Effect of miR-497 on myocardial cell apoptosis in rats with myocardial ischemia/reperfusion through the MAPK/ERK signaling pathway

K. JIN, B. WANG, Z.-B. RUAN, G.-C. CHEN, Y. REN

Department of Cardiovascular Medicine, Taizhou People's Hospital, Taizhou, China

Kai Jin and *Bin Wang* contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of micro ribonucleic acid (miR)-497 on myocardial cell apoptosis in rats with myocardial ischemia/reperfusion (I/R) through the mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (ERK) signaling pathway.

MATERIALS AND METHODS: A rat model of myocardial I/R was established, myocardial cells were extracted, and miR-497 was inhibited by inhibitors and overexpressed using miRNA mimics. The cell apoptosis rate was detected by flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The interaction between miR-497 and ERK was determined by dual-luciferase reporter gene assay. The change in the protein level was measured via Western blotting (WB).

RESULTS: Up-regulation of miR-497 promoted myocardial cell apoptosis, and the 3'-untranslated region (3'-UTR) of ERK was highly conserved to combine with miR-497. The luciferase reporter gene assay showed that the transfection of miR-497 could significantly inhibit the relative luciferase activity in cells.

CONCLUSIONS: MiR-497 overexpression significantly down-regulated the ERK expression at messenger RNA (mRNA) and protein levels in cells. MiR-497 plays an important role in regulating I/R injury-induced myocardial cell apoptosis by targeting the ERK-induced apoptosis pathway.

Key Words:

MiR-497, MAPK/ERK signaling pathway, Myocardial ischemia/reperfusion, Myocardial cell, Apoptosis.

Introduction

The prevalence rate of ischemic heart disease is increasing worldwide and is expected to become the main and most common threat to human life by 2020[1]. Myocardial ischemia/reperfusion (I/R) injury is one of the causes of ischemic events in patients with heart disease. The characteristics of I/R injury include myocardial cell necrosis, apoptosis, mitochondrial dysfunction, and elevated lipid peroxide. I/R injury easily causes myocardial dysfunction, including the malignant arrhythmia, reduced left ventricular systolic function, and decreased ventricular pressure[2]. Therefore, it is urgent to put forward an effective I/R injury prevention strategy.

A micro ribonucleic acid (miRNA) is a kind of endogenous, small, non-coding single-stranded RNA, which negatively regulates gene expression through combining with the 3'-untranslated region (3'-UTR) of the targeted messenger RNA (mRNA) and affects subsequent translation, inhibition or targeted gene degradation. MiRNAs participate in most major cell functions, such as cell differentiation, growth, migration, and death[3]. MiRNAs respond to different cardiac pressures such as myocardial I/R through upward or downward regulation, so that miRNAs are defined as key regulators of cardiovascular diseases, including I/R injury[4-6]. For example, miR-1 and miR-133 negatively regulate cardiac hypertrophy, while miR-195 stimulates pathological hypertrophy and heart failure[7]. MiR-21 regulates the growth and survival of myocardial cells and fibroblasts, thus actively mediating myocardial hypertrophy[8,9]. However, more and more evidence confirms the important role of miR-497 in many activities such as tumor occurrence and metastasis as well as cell proliferation and apoptosis[10,11]. MiR-497, as a tumor suppressor, has not yet been reported for its role in heart I/R injury.

In this study, it was found that miR-497 overexpression could promote ischemic myocardial injury in rat models, and hypoxia/reoxygena-

Corresponding Author: Yin Ren, MM; e-mail: renyinmail@163.com

tion (H/R) could stimulate apoptosis of cultured rat myocardial cells. Also, this study demonstrated for the first time that miR-497 could directly target the mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (ERK) signaling pathway to regulate its expression and promote cell apoptosis. To sum up, these findings suggest that miR-497 may be a new therapeutic target for ischemic heart disease.

Materials and Methods

Establishment of the Animal Model of I/R

A total of 30 healthy male C57BL/6 rats (8-10 weeks old) weighing 20-30 g were kept in an environment chamber with a constant temperature of 20°C, a humidity of 60% and a 2:12 h dark/light cycle for circadian rhythm control. All rats were purchased from Beijing Laboratory Animal Center (Beijing, China). Animal experiments were approved by the Laboratory Animal Ethics Committee of Taizhou People's Hospital (Taizhou, People's Republic of China). All rats were free to drinking water and having a sterile standard diet. The rats were randomly divided into two groups, namely, Sham operation group (n=15) and I/R group (n=15). The rats in Sham operation group received sham operation for 150 min, with enlarged heart, sutured left anterior descending artery (LAD) and non-ligated coronary artery. The rats in I/R group underwent reversible LAD ligation, and I/R was induced by ischemia for 30 min, followed by reperfusion for 2 h.

Establishment of the I/R Injury Model

All rats were anesthetized with pentobarbital sodium (50 mg/kg) for intraperitoneal injection and fixed for endotracheal intubation using a small animal respirator. After hair was removed from the precordial region, the skin was disinfected with iodine. A longitudinal incision was made between the 3rd and 4th rib to separate the pectoralis major muscle, and the serrated anterior muscle was separated from the pectoralis minor muscle to expose the heart. A 6-0 nylon suture was then placed around 1-2 cm at the root of the LAD and loosened after 30 min of occlusion, and the LAD was perfused for 2 h. The heart of all rats was then separated and prepared for subsequent experiments, and the electrocardiogram (ECG) was recorded synchronously until the end of the experiment.

Determination of the Infarction Area (IA)

The IA of the myocardium was measured by triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) staining. The heart was perfused with saline on the Langendorff system to flush blood from the coronary vasculature. 2% Evans blue dye (wt/vol, Sigma-Aldrich, St. Louis, MO, USA) was injected into the vena cava to identify the area of myocardial perfusion at the end of myocardial reperfusion. The left ventricle was separated through the remove of the right ventricle and the flushing of the remaining blood. The samples were then sliced and stained with 1% TTC at 37°C for 10 min. The red area was identified as the area at risk (AAR), while the IA was identified by the non-stained (white) area. AAR and IA were determined by computerized surface methodology. Finally, the percentage of IA/AAR was calculated.

Cell Transfection

MiR-497/negative control (NC) mimics and miR-497/NC inhibitors were synthesized by GenePharma (Shanghai, China). Cell transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from cultured cells or fresh surgical tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), quantitative RT-PCR (qRT-PCR) was carried out using the All-in-One miRNA qRT-PCR detection kit for miR-497 (GeneCopoeia, USA), and the relative expression level of mRNA was measured by SYBR Green qRT-PCR assay (Bio-Rad Laboratories Inc., Hercules, CA, USA), with β-actin as an endogenous control. All qRT-PCR were performed on an ABI 7500 thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences are shown in Table I.

Detection of Cell Apoptosis Via Flow Cytometry

After different treatment, cell culture media were collected into centrifuge tubes, respectively. Cells were washed with phosphate-buffered saline (PBS) twice and moderately digested with trypsin. The digestion of all the cells in each group was terminated with the culture solution collected before, and after beating, the cells were collected into centrifuge tubes for centrifugation at 1000

Table I. Primer sequences.

Gene	Primer sequence
miR-497	Forward: 5'-TGCGGAAAAGCTGGGTTGAGAG-3' Reverse: 5'-CCAGTGCAGGGTCCGAGGT-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3' Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
ERK	Forward: 5'-ATGCCAGAGGAAGGAGGAGC-3' Reverse: 5'-GAGCCCACAGTGACAGAATAGG-3'
β-actin	Forward: 5'-AGTGTGACGTGGACATCCGCAAAG-3' Reverse: 5'-ATCCACATCTGCTGGAAGGTGGAC-3'

rpm for 5 min. The supernatant was discarded, and 1 mL precooled PBS solution was added to resuspend the cells. After centrifugation at 1000 rpm for 5 min, the supernatant was discarded, 50 μL remaining solution was reserved. Subsequently, all the cells were transferred to 1.5 mL Eppendorf (EP) tubes and repeatedly washed with PBS solution, and the supernatant was discarded. The apoptosis level of each group was detected using the BD Annexin V-FITC Apoptosis Kit (San Jose, CA, USA) and analyzed by flow cytometry on BD FACSCalibur (Becton Dickinson Co., Franklin Lakes, NJ, USA).

Western Blotting (WB)

The bicinchoninic acid (BCA) protein concentration determination kit was used to determine the protein concentration (Beyotime, Shanghai, China). 40 μg total proteins were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Then, the cells were incubated with the first antibody (1:2000, Abcam, Cambridge, UK), and the corresponding horseradish peroxidase-labeled secondary antibody (1:1000) was added for incubation at room temperature for 1 h (Beyotime, Shanghai, China). 200 μL luminescent liquid was evenly added dropwise and placed in a chemiluminescence imaging analysis system for development. ImageJ software was adopted to calculate the gray value of bands on the developed image, which was then statistically analyzed.

Dual-Luciferase Reporter Gene Assay

On the first day of the experiment, cells were digested and inoculated (appropriate cells were selected according to the specific experiment) in a 35 mm cell culture dish and cultured overnight in an incubator with 5% CO_2 and saturated humidity at 37°C. When cell density reached 70%, myocardial cells were co-transfected with 0.2 μg luciferase reporter vectors and miR-497 mimics or control mimics. The assay was standardized with 0.05 μg red fluorescent protein expression vector pDsRed2-N1 (Clontech, USA). After 48 h, the cells were lysed with RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris/HCl, pH: 7.2, 1% Triton X-100 and 0.1% SDS). The fluorescence intensity of luciferases and red fluorescent proteins was detected by an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The difference between the two groups was detected *via* the *t*-test, and the intergroup differences among more than two groups were analyzed by ANOVA. Bilateral 95% confidence interval (CI) was used in all tests, and $p<0.05$ represented that the difference was statistically significant.

Results

Expression of MiR-497 in I/R Injury Model and Control Group

The degree of myocardial infarction induced by I/R injury was verified by the ratio of IA/AAR. The results showed that the IA of I/R group was significantly larger than that of Sham operation group ($p<0.05$) (Figure 1A). The influence of I/R injury on myocardial cell apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, which manifested that the proportion of TUNEL-positive myocardial cells in I/R group was evidently higher than that in Sham operation group ($p<0.05$) (Figure 2). The relative level of miR-497 in myo-

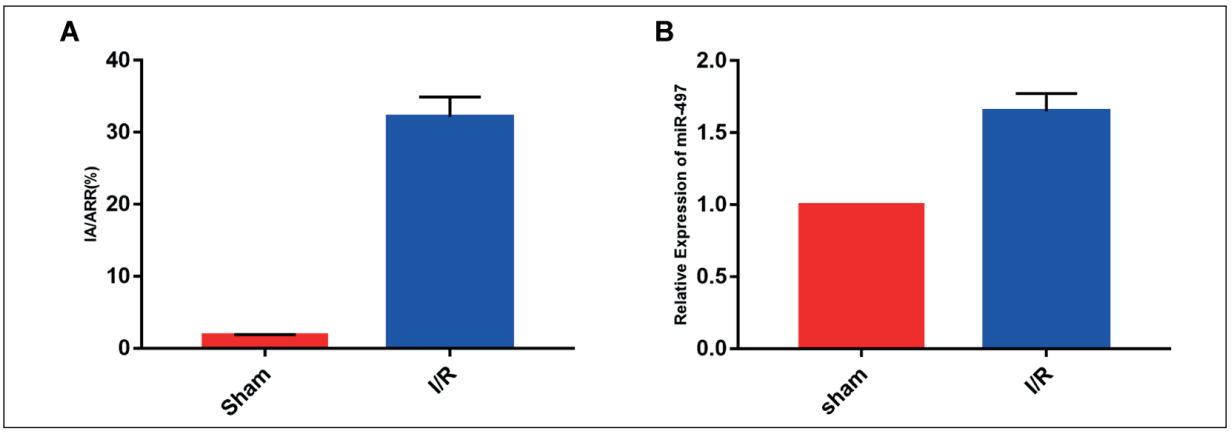

Figure 1. A, IA of I/R group is significantly larger than that of Sham operation group ($p<0.05$). **B,** MiR-497 expression in the I/R injury model and normal model: The relative level of miR-497 in myocardial tissues after I/R injury is evidently higher than that in Sham operation group ($p<0.05$).

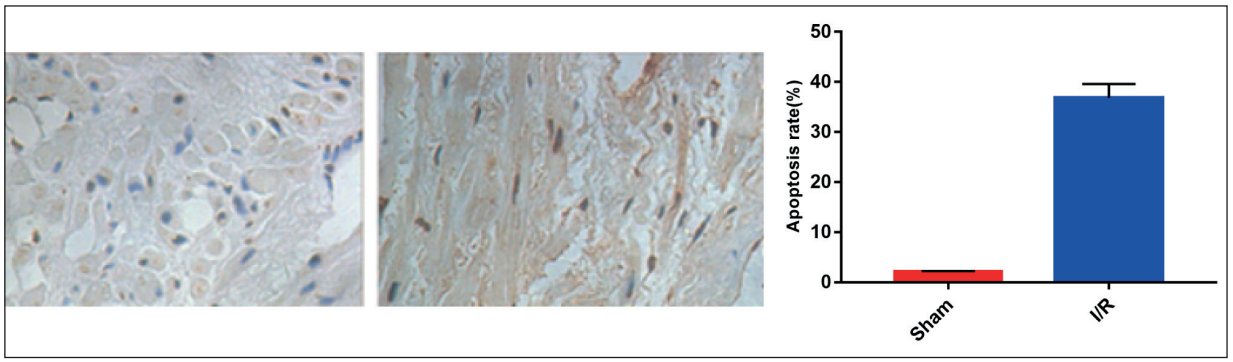

Figure 2. Influence of I/R injury on myocardial cell apoptosis detected by TUNEL assay (magnification: 100×). The proportion of TUNEL-positive myocardial cells in I/R group is remarkably higher than that in Sham operation group ($p<0.05$).

cardial tissues after I/R injury was notably higher than that in Sham operation group ($p<0.05$) (Figure 1B).

Cell Transfection

To identify the effect of miR-497 on myocardial cell apoptosis induced by H/R injury, miR-497 mimics, anti-miR-497 mimics, and the corresponding negative controls were transfected into myocardial cells after H/R injury. QRT-PCR results manifested that the miR-497 expression was markedly increased after transfection with miR-497 mimics, while anti-miR-497 mimics decreased the miR-497 expression in myocardial cells (Figure 3).

Detection of Cell Apoptosis

Flow cytometry was applied to detect apoptosis in different groups. Compared with controls, the up-regulation of miR-497 promoted myocardial cell apoptosis. The apoptosis rates of con-

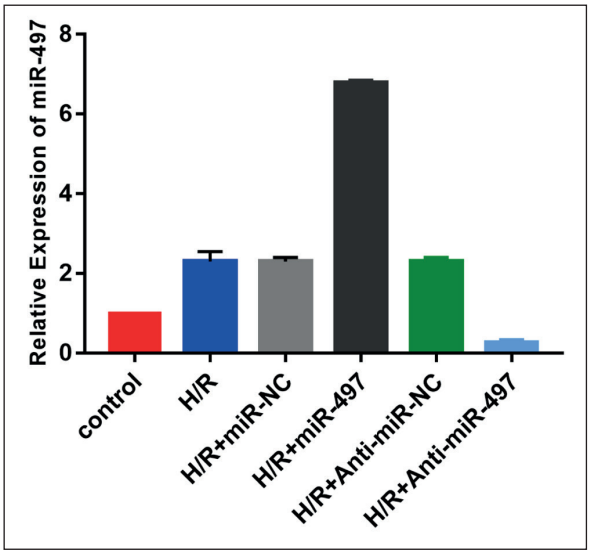

Figure 3. Expression levels of miR-497 after different treatments detected *via* qRT-PCR. Expression level of miR-497 is up-regulated after transfection with miR-497 mimics but down-regulated after transfection with miR-497 inhibitors ($p<0.01$).

trol group, H/R+miR-control group, H/R group, and H/R+miR-497 group were (4.68±2.62)%, (12.77±1.28)%, (13.42±1.35)%, and (16.15±2.62)%, respectively, displaying statistically significant differences ($p<0.05$). These data indicate that miR-497 mediates the apoptosis of myocardial cells after H/R injury (Figure 4-5).

Dual-Luciferase Reporter Gene Assay

TargetScan and PicTar online tools were utilized to determine miR-497 targets and identify the conserved binding sites of miR-497 in the 3'-untranslated region (3'-UTR) of ERK genes. The predicted target of miR-497 was examined. It was found that ERK's 3'-UTR was highly conserved to combine with miR-497. The dual-luciferase reporter gene assay revealed that transfection of miR-497 markedly inhibited the relative luciferase activity in cells, indicating that miR-497 inhibits the ERK expression by interacting with ERK's 3'-UTR (Figure 6).

MiR-497 Overexpression Inhibited the MAPK/ERK Signaling Pathway

Overexpression of miR-497 notably down-regulated the ERK expression at mRNA and protein levels in cells (Figure 7). After cell transfection with miR-497 mimics for 48 h, the expression of ERK mRNAs and proteins in different groups showed statistical differences. Compared with controls, miR-497 overexpression decreased the ERK expression.

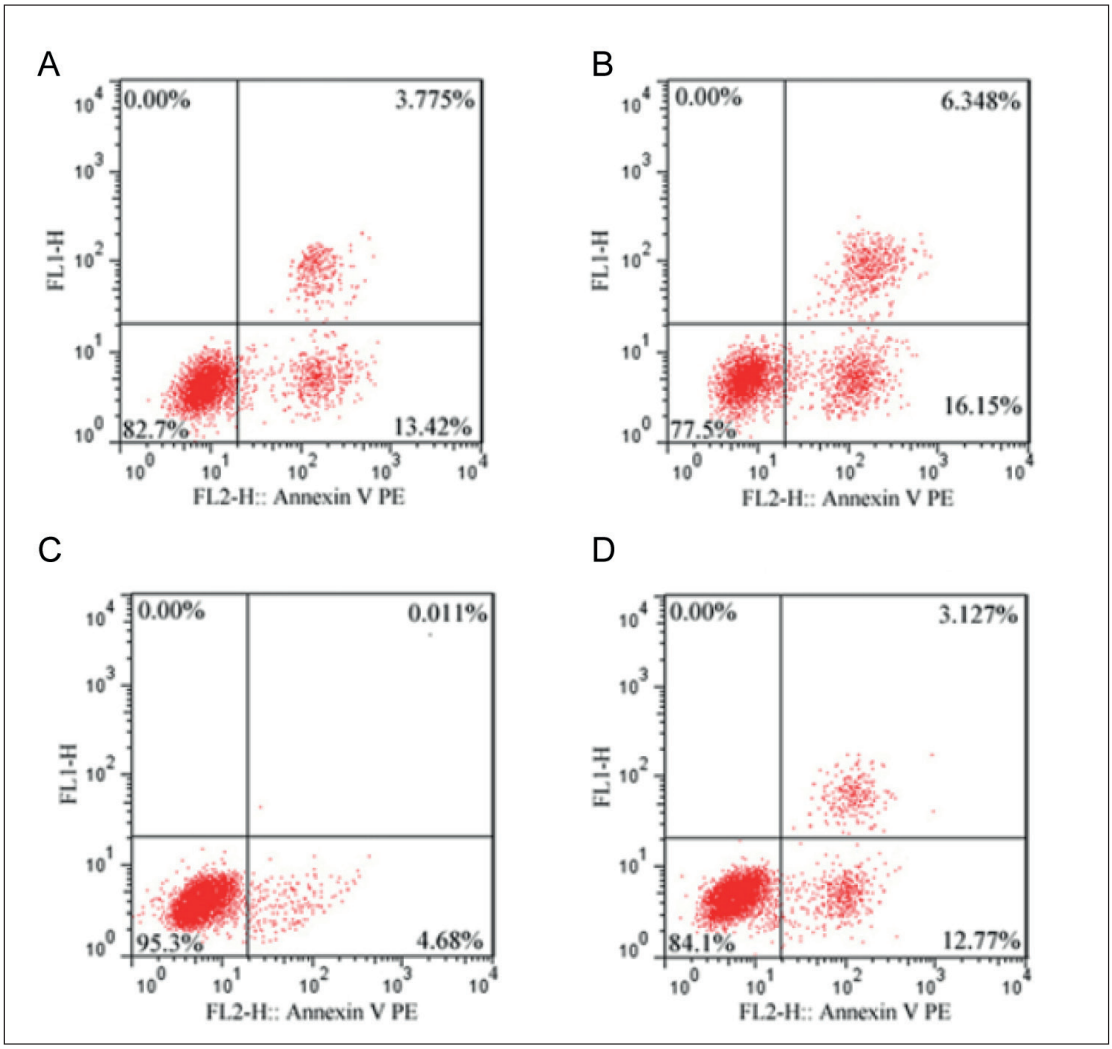

Figure 4. Apoptosis rate detected by flow cytometry. **A,** Control group, **B,** H/R + miR-control group, **C,** H/R group, and **D,** H/R + miR-497 group.

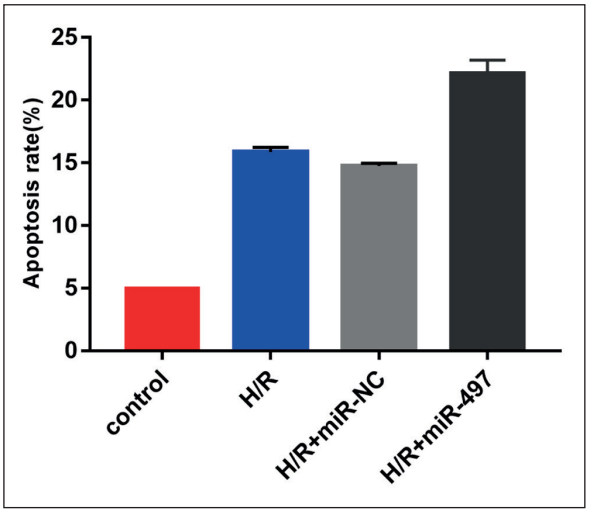

Figure 5. Cell apoptosis detected *via* flow cytometry. The apoptosis rates of control group, H/R+miR-control group, H/R group, and H/R+miR-497 group are (4.68±2.62)%, (12.77±1.28)%, (13.42±1.35)%, and (16.15±2.62)%, respectively ($p<0.05$).

Discussion

Recently, miRNAs have been confirmed to be related to I/R injury-induced myocardial apoptosis and regulation of cardiac function[12]. However, the function and mechanism of these maladjusted miRNAs in I/R injury-induced myocardial cell apoptosis are still not defined. It is reported that various miRNAs, such as miR-1, miR-146a, miR-199a-3p, and miR-214, are out of balance in myocardial I/R injury related to cell proliferation, apoptosis, and death[13-15]. In this investigation, a rat model of myocardial I/R injury was initially established, and it was found that the expression of miR-497 and the apoptosis rate of myocardial cells in I/R injury group were significantly higher than those in H/R group and the corresponding control group. In addition, it was observed that ERK is the functional target of miR-497 in myocardial cells. Therefore, the findings in this work proved that miR-497 might be the promoter of myocardial cell apoptosis induced by myocardial I/R injury, and abnormal changes in miR-497-ERK interaction might lead to the occurrence and development of I/R injury.

Previous studies have shown that miR-497 targets anti-apoptosis gene b-cell lymphoma 2 (Bcl-2) and autophagy gene microtubule-associated proteins 1A/1B light chain 3B (LC3B). In non-myocardial cells, miR-497 promotes the death of ischemic neurons by down-regulating Bcl-2 and Bcl-w and acts as a tumor suppressor to inhibit the occurrence of tumors. The overexpression of miR-497 in human neuroblastoma cells can increase the formation of reactive oxygen species, destroy the mitochondrial membrane potential, and induce the cytochrome C release[16]. Since apoptosis and autophagy are closely related to myocardial I/R injury, this study was designed to explore the effect of miR-497 on myocardial cell fate during H/R or IR injury. Data in this study indicate that miR-497 is a new therapeutic target for myocardial injury.

A miRNA is a small non-coding regulatory RNA that regulates target genes extensively by binding to complementary sequences in 3'-UTR. Therefore, establishing the relationship between miRNAs and their target genes can better help the understanding of the molecular mechanism of the progression of myocardial I/R injury and the potential therapeutic targets for clinical treatment of myocardial I/R injury. We suggest that miR-497

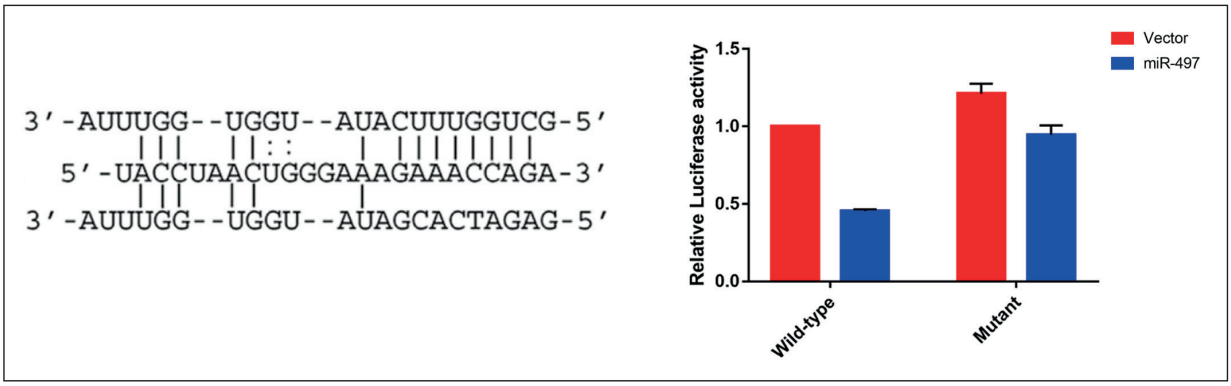

Figure 6. MiR-497 directly targets ERK: predicted binding sequence of miR-497 in ERK 3'-UTR and interaction between miR-497 and ERK determined by luciferase reporter gene assay.

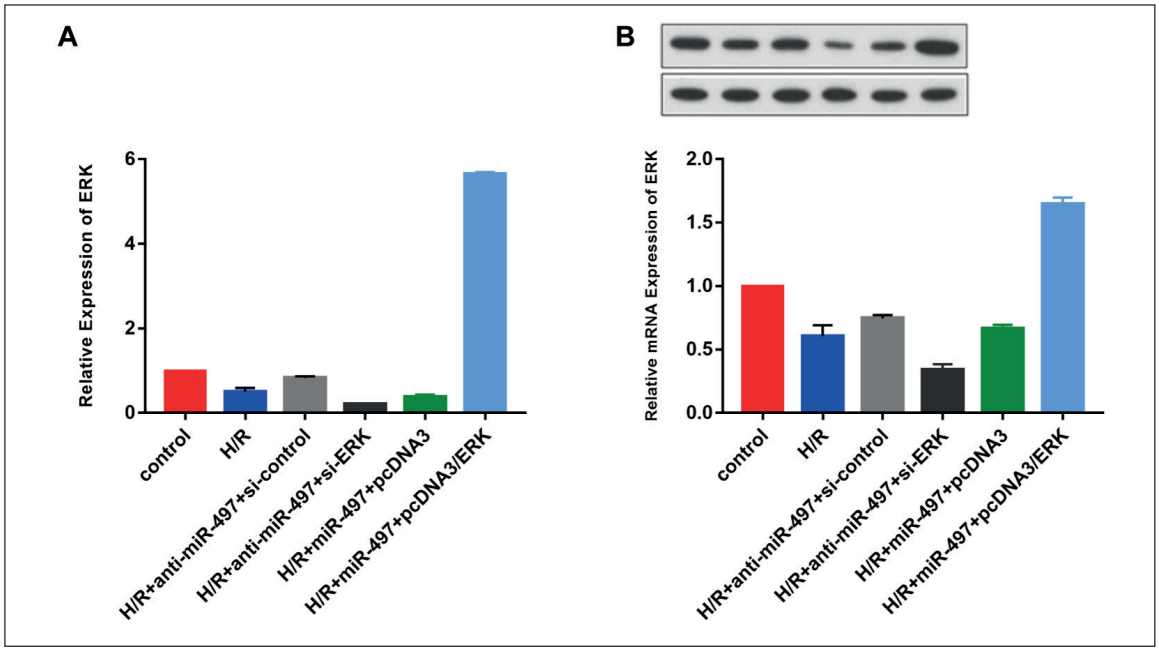

Figure 7. MiR-497 down-regulates the ERK expression in cells. After miR-497 overexpression, the mRNA and protein levels of ERK are decreased.

has a negative regulatory effect on ERK mRNA and protein levels in myocardial cells. Also, the direct targeting effect is further supported by luciferase report analysis.

Cell apoptosis is considered to be an important mechanism of myocardial injury. It is reported that several important molecules are involved in the progress of apoptosis, including Fas, Fasl, and Caspase-3[17]. Moreover, the mitochondria of myocardial cells are abundant, accounting for about 30% of the weight of the myocardium[18]. Therefore, oxidative stress is the main cause of heart failure, myocardial I/R, myocardial infarction and other cardiac diseases[19]. MAPKs, namely, p38 MAPK, ERK1/2, and JNK, are activated by stresses in different cellular systems, including I/R[20]. Previous studies have revealed that ERK1/2 is activated a few minutes before reperfusion and provides cardiac protection against oxidative stresses by blocking apoptosis[21,22]. Some research results denoted that the MAPK signaling pathway is involved in apoptosis attenuation and Caspase-3 activation after I/R. The ERK1/2 pathway is involved in the cardioprotective effect, which is further proved by the U-0126 (an upstream inhibitor of ERK1/2)-induced cardiac function recovery, reduced infarction size, decreased enzyme release, decreased apoptosis, and decreased Caspase-3 activity.

The down-regulation of myocardial ischemia or I/R by miR-497 may be an important adaptation mechanism for up-regulating the expressions of Bcl-2 and LC3B. The silencing of endogenous miR-497 provides protection against I/R-induced myocardial cell death and apoptosis by targeting Bcl-2 and LC3B. Therefore, miR-497 and its downstream targets can be used as valuable therapeutic targets to improve cardiac performance after I/R injury. Since a single miRNA can regulate multiple different mRNAs, and the same mRNA can be silenced by multiple miRNAs, it is of great significance to further explore the targeting network of miR-497 in ischemic diseases. As the complementary sites on lncRNAs enable it to recognize and bind miRNAs and act as a highly specific sensor for regulating miRNAs, the recognition of lncRNAs that regulate miR-497 will also help to find new therapeutic targets for myocardial I/R injury.

Conclusions

We showed that miR-497 plays an important role in regulating I/R injury-induced myocardial cell apoptosis by targeting the ERK-induced apoptosis pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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