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Overexpression of miR-431 inhibits cardiomyocyte apoptosis following myocardial infarction *via* targeting HIPK3

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Abstract. – OBJECTIVE: Acute myocardial infarction (AMI) is a common acute cardiovascular crisis. Although the diagnosis and treatment of AMI is constantly improving, the mortality of AMI are still very high, and its pathogenesis is still unclear. This article focuses on the role of microRNA-431 (miR-431) in regulating myocardial apoptosis after myocardial infarction (MI) and its potential molecular mechanism.

MATERIALS AND METHODS: We constructed cell models and animal models of MI. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was used to detect miR-431 expression in myocardium after MI. Western blot, cell counting kit-8 (CCK-8) assay, flow cytometry and terminal dexynucleotidyl transferase(TdT)-mediated dUTP nick end labeling (TUNEL) staining were performed to detect myocardial apoptosis; pathological sections of myocardium, serum lactate dehydrogenase (LDH) levels and Caspase-3 activity in myocardium were employed to evaluate myocardial injury of MI rats; echocardiography was utilized to assess cardiac function of rats.

RESULTS: We revealed that miR-431 expression was decreased in H2O2-treated H9c2 cells and myocardium of MI rats. The expression of Cleaved Caspase-3 (C-Caspase-3) in H9c2 cells treated with H2O2 was significantly increased, the cell viability was dramatically decreased, the apoptosis rate and the percentage of TUNEL positive cells were notably increased, but up-regulation of miR-431 could reverse these effects. At the same time, compared with the sham group, serum LDH levels were observably increased, myocardial Caspase-3 activity was also increased, and cardiac function was greatly reduced, while overexpression of miR-431 could reduce myocardial injury and improve cardiac function of MI rats. Through the Luciferase reporter gene experiment, we found that miR-431 could directly target HIPK3.

CONCLUSIONS: In summary, overexpression of miR-431 can inhibit apoptosis after myocardial infarction *via* targeting HIPK3, thereby reducing myocardial injury and improving cardiac function in MI rats.

Key Words:

Acute myocardial infarction, MicroRNA-431, Apoptosis, HIPK3.

Introduction

Acute myocardial infarction (AMI) is caused by various factors that lead to a sharp reduction or sudden interruption of coronary blood flow. The myocardium undergoes severe and persistent acute ischemia, which causes myocardial ischemic necrosis[1,2]. After MI, myocardial cell apoptosis and necrosis due to ischemia and hypoxia lead to cardiac dysfunction, which may eventually lead to heart failure. In clinical treatment, interventional therapy, coronary artery bypass grafting, and other methods are often used for patients with MI, which can effectively prevent the enlargement of the infarct area. At the same time, drug treatment needs to be strengthened[3,4]. Therefore, we should start from a multi-dimensional perspective, look for new effective drugs and therapeutic targets, and provide new strategies for the prevention and treatment of MI with multiple targets and multiple pathways.

MicroRNA (miRNA) is a type of non-coding single-stranded RNA molecule found in eukaryotic cells that is encoded by endogenous genes and has a length of approximately 22 nucleotides. Through the regulation of its target gene transcription, it participates in the regulation of eukaryotic cell differentiation, proliferation, growth and apoptosis. In cardiovascular disease, miRNA not only plays a decisive role in myocardial hypertrophy, myocardial necrosis and apoptosis by regulating its target genes, but also participates in each process of AMI[5-8]. Recent studies[9-11] have found that miR-431 is involved in the regulation of the pathophysiological processes of various tumors, including apoptosis, proliferation, and mi-

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gration. MiR-431 has also been reported to reduce cerebral ischemia-reperfusion injury by promoting proliferation and inhibiting apoptosis of hippocampal neuron[12]. However, the role of miR-431 in myocardial infarction has not been studied.

In this report, we constructed a cell model and animal model of MI to verify the regulatory effect of miR-431 on myocardial cell apoptosis after MI from the cellular level and animal level, respectively. Our results reveal that up-regulation of miR-431 may be a potential target for the treatment of MI.

Materials and Methods

Rat MI Model

Thirty adult male Sprague Dawley (SD) rats weighing 200-250 g and aged 8-10 weeks were purchased from Animal Center of Chinese Academy of Medical Sciences. The rats were reared at an ambient temperature of about 25°C with 12 h/12 h light conditions. Rats were kept for one week before modeling to adapt to the environment. All animal experiments were in compliance with the "Regulations for the Management of Laboratory Animals" and animal ethics requirements. This investigation was approved by the Animal Ethics Committee of Cangzhou Central Hospital Animal Center.

One day before the operation, 10 rats were injected with miR-431 agomiR (agomiR-431, 5 mg/kg) (RiboBio, Guangzhou, China) through the tail vein to increase the expression of miR-431 in rat myocardium, and 10 rats were injected with agomiR-431 negative control (NC) through the tail vein. The agomiR-431 was the same double-stranded RNA analog as mature rno-miR-431, whose structure was chemically modified and bound to cholesterol molecules to be used *in vivo*. The rats in the sham group only had thoracotomy but no ligation. So, the rats were divided into three groups: sham group, MI + NC group, MI + agomiR-431 group.

Rats were intraperitoneally injected with 5% pentobarbital sodium (40 mg/kg) for anesthesia, placed in a supine position and fixed on the operating table. The heart was exposed after thoracotomy at the left third intercostal space, and the pericardium was opened. The left anterior descending coronary artery was ligated with a 6-0 suture (the needle was inserted 3 mm below the root of the left atrial appendage and the needle was discharged at the edge of the pulmonary artery cone). The color of the ventricular muscle below the ligation site was whitish, the movement of the ventricular wall was weakened, and the T wave high tip and the ST segment of the ECG lead II continued to arch upward, indicating that the model was successfully made and the ligation time was recorded. After operation, rats were injected with 100,000 units of penicillin intraperitoneally to prevent wound infection.

Cell Culture and Transfection

H9c2 cells (Invitrogen, Carlsbad, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA), which contained 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). H9c2 cells were cultured in an incubator at 37°C and containing 5% CO_2, and the medium was changed every 2 days.

MiRNA mimic is synthesized using chemical methods to mimic the high-level expression of mature miRNA in cells. MiR-431 mimic or mimic negative control (NC) (RiboBio, Guangzhou, China) was transfected into H9c2 cells to study the function of miR-431 *in vitro*. 48 hours later, H9c2 cells were treated with H_2O_2 (100 μM, 4 h) to establish cell model of MI.

Echocardiographic Measurement

On the 7th day after the operation, 6 rats were taken from each of the three groups. After anesthetizing the rats with isoflurane, the ultrasonography probe was used to detect the M-ultrasound image on the long axis of the left ventricle next to the sternum. The left ventricular end-systolic diameter and left ventricular end-diastolic diameter were measured, and the left ventricular ejection fraction (EF) and left ventricular short axis shortening rate (FS) were calculated.

Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis

H9c2 cells were collected and total RNA was extracted using TRIzol kit (Invitrogen, Carlsbad, CA, USA). Rat hearts were collected on the 1st, 3rd, and 7th days of surgery. The myocardium in infarcted zone, in border zone, and in remote zone of the left ventricular were separated. Total RNA of myocardium was also extracted using TRIzol kit. MiRNA reverse transcription was performed using MicroRNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the protocols. A 20 μL RT-qPCR reaction system was prepared using SYBR Premix ExTaq™ II (TaKaRa, Otsu, Shiga, Japan), and complementary deoxyribose nucleic acid (cDNA) amplification was performed using an ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA). PCR parameter setting: 95°C -30 s, 90°C -5 s, 65°C -30 s, 40 cycles in total. U6 is the internal reference of miRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is the internal reference of mRNA. All the primers were listed in Table I.

Western Blot

H9c2 cells were collected and total protein was extracted using radioimmunoprecipitation assay (RIPA) Lysis Buffer (Beyotime Biotechnology, Shanghai, China). The protein concentration was detected using the bicinchoninic acid (BCA) assay kit (Beyotime Biotechnology, Shanghai, China). A corresponding amount of 5 × Loading Buffer (Beyotime Biotechnology, Shanghai, China) was added to the protein sample and heated for 5 minutes in a 95°C dry thermostat to denature the protein. After that, the protein sample was stored at a temperature of -20°C.

According to the molecular weight of the protein, a certain concentration of separation gel was prepared. After the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared, the protein marker and samples were added in the order of grouping. The total protein amount in each lane was 50 μg. The electrophoresis instrument was adjusted to a voltage of 100 V. After the protein marker was separated, it was adjusted to 200 V. When the phenol blue moved to 1 cm from the bottom of the gel, the electrophoresis was stopped. When the electrophoresis was complete, the electrophoresed protein was transferred to the polyvinylidene difluoride (PVDF, EpiZyme, Shanghai, China) membrane. After the transfer was stopped, the PVDF membrane was blocked with 5% skim milk at room temperature for 2 hours. The blocked PVDF membrane was washed 3 times with Tris Buffered Saline and Tween-20 (TBST), each time for 5 minutes. Then, the PVDF membrane was incubated at 4°C overnight with a diluted primary antibody (HIPK3, Abcam, Cambridge, MA, USA, Mouse, 1:1000; Caspase-3, Abcam, Cambridge, MA, USA, Mouse, 1:1000; Cleaved Caspase-3, Abcam, Cambridge, MA, USA, Mouse, 1:1000; GAPDH, Abcam, Cambridge, MA, USA, Mouse, 1:1000). After that, the PVDF membrane was washed with TBST for 3 times, each time for 5 minutes. At room temperature, the PVDF membrane was incubated with the diluted secondary antibody for 1.5 h, and then washed with TBST for 3 times, each time for 5 minutes. Finally, the PVDF membrane was placed in the prepared enhanced chemiluminescence (ECL) developer and soaked thoroughly, and then photographed and preserved using the gel imaging system.

Cell Counting Kit-8 (CCK-8) Assay

The logarithmic growth phase of H9c2 cells was seeded in a 96-well culture plate with a cell suspension of 5×10^4 cells/mL. After 24 hours of cell culture, 10 μL of CCK-8 reagent (Beyotime Biotechnology, Shanghai, China) was added. One hour later, the optical density (OD) value at a suitable excitation wavelength was detected using Microplate Reader.

Flow Cytometry

H9c2 cells in good growth state were seeded in 6-well plates and transfected with miR-431 or NC for 48 hours, and then treated with H_2O_2 for 4 hours. Trypsin was used to digest H9c2 cells. The cells were centrifuged to remove supernatant and washed 1-2 times with PBS. 500 μL Binding buffer was added to each group of cells and mixed gently. Then, 5 μL of Annexin V-FITc (KeyGen, Shanghai, China) and PI (KeyGen, Shanghai, China) were added. Finally, the mixed solution was incubated at room temperature in the dark for 10-20 minutes, and then analyzed by flow cytometry.

Table I. Real time PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
miR-431	GTGTCTTGCAGGCCGT	GTTGTGGTTGGTTGGTTTGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
HIPK3	CCCAGCAACCGAAGTTT	GTGTTCGCTCCCATCAAT
GAPDH	ATGGCTACAGCAACAGGGT	TTATGGGGTCTGGGATGG

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

Terminal Dexynucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining

Four groups of cells were fixed with 4% paraformaldehyde. The permeability of cell membrane was increased using 0.1% Triton X-100. Then, the prepared TUNEL reagent (Roche, Basel, Switzerland) was co-incubated with the cells. 4',6-diamidino-2-phenylindole (DAPI) (Roche, Basel, Switzerland) was used to stain the nucleus. The results were observed with a fluorescence microscope.

Hematoxylin-Eosin (HE) Staining

On the 7th day after surgery, the rat hearts were collected, and the hearts were soaked with 10% neutral formalin fixative. Afterwards, different concentrations of alcohol are used to dehydrate the heart tissue gradient. Afterwards, the heart was embedded in paraffin and sliced. The paraffin sections were then dewaxed and stained, then mounted with neutral resin and observed under a microscope.

Lactate Dehydrogenase (LDH) Contents and Caspase-3 Activity

On the 7th day after the operation, the blood and heart tissue of the rats were collected. Serum LDH contents and Caspase-3 activity of infarcted myocardium were detected by LDH ELISA kit (Dojindo Molecular Technologies, Shanghai, China) and Caspase-3 activity detection kit (Beyotime Biotechnology, Shanghai, China), respectively.

Luciferase Activity Assay

Luciferase reporters (RiboBio, Guangzhou, China) containing wild-type (HIPK3-WT) or mutant (HIPK3-MUT) 3'UTR of HIPK3 were constructed. MiR-431 mimic or NC along with Luciferase reporters were co-transfected into HEK293T cells. Luciferase activities were detected using the Dual-Luciferase Reporter Assay System kit (RiboBio, Guangzhou, China).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22. 0 software (IBM, Armonk, NY, USA). Data were represented as mean ± Standard Deviation (SD). The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

MiR-431 Expression Was Reduced in Infarcted Myocardium

MiR-431 expression was markedly decreased in H9c2 cells treated with H_2O_2 (Figure 1A). In addition, miR-431 expression was observably decreased in infarcted zone and border zone of myocardium in MI rats, but there was no significant change in remote zone (Figure 1B~1D). Later, to study the function of miR-431, miR-431 mimic was transfected into H9c2 cells to increase miR-431 expression *in vitro* (Figure 1E). The expression of miR-431 in rat myocardium was also significantly increased by injection of agomiR-431 through the tail vein (Figure 1F). The expression of miR-431 in cell models and animal models indicated that miR-431 expression decreased in MI.

Overexpression of MiR-431 Inhibited H_2O_2-Induced Cardiomyocyte Apoptosis

We detected the expression of Caspase-3 and Cleaved Caspase-3 (C-Caspase-3) in the cells of each group. Compared with the control group, the ratio of C-Caspase-3 / Caspase-3 in the H_2O_2 group was remarkably increased. When the cells were transfected with miR-431 mimic, the ratio of C-Caspase-3 / Caspase-3 was significantly reduced (Figure 2A). Furthermore, overexpression of miR-431 can reverse the H_2O_2-induced decrease in H9c2 cell viability (Figure 2B). Through flow cytometry, we found that H_2O_2 treatment can significantly induce apoptosis of H9c2 cells. However, after the cells were transfected with miR-431 mimic, the apoptosis rate was significantly reduced (Figure 2C). The results of the TUNEL staining are similar. Up-regulation of miR-431 can significantly protect H9c2 cells from H_2O_2-induced apoptosis (Figure 2D). Based on these results, we conclude that up-regulation of miR-431 can inhibit H_2O_2-induced apoptosis of H9c2 cells.

Overexpression of MiR-431 Alleviated Myocardial Damage and Improved Cardiac Function in MI Rats

From HE staining of myocardium, we found that the myocardial fibers in the sham group were neatly arranged, the intima cells and the myocardium were intact, the cytoplasm was uniformly stained and the overall shape was normal. Howev-

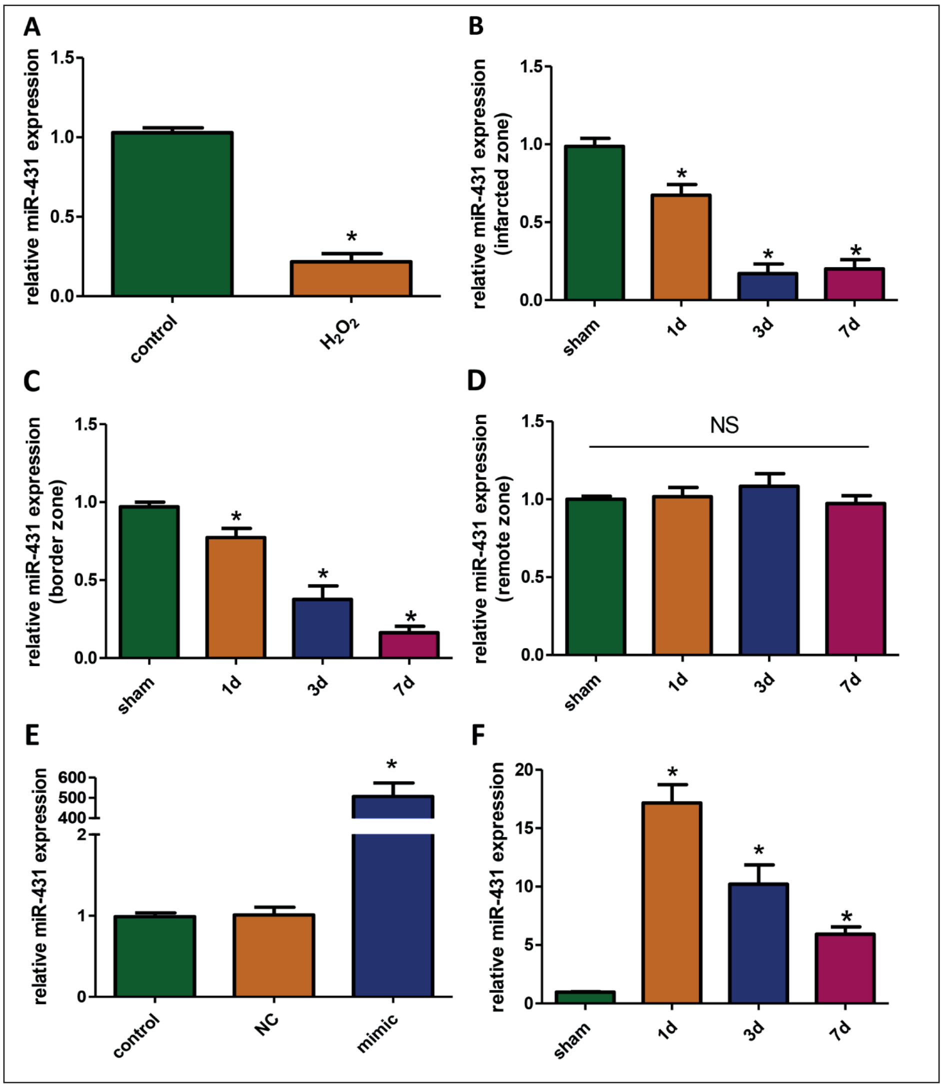

Figure 1. MiR-431 expression was reduced in infarcted myocardium. **A**, The expression of miR-431 in H9c2 cells treated with H_2O_2 was detected by RT-PCR (“*” $p<0.05$ *vs.* control, n = 3). The expression of miR-431 in infarcted zone (**B**), border zone (**C**) and remote zone (**D**) of hearts of MI rats were detected by RT-PCR (“*” $p<0.05$ *vs.* sham, n = 3). **E,** Transfection of miR-431 mimic into H9c2 cells significantly increased miR-431 expression (“*” $p<0.05$ *vs.* control, n = 3). **F,** RT-PCR analysis showed that the tail vein injection of agomiR-431 increased the expression of miR-431 in rat heart (“*” $p<0.05$ *vs.* sham, n = 3).

er, in the MI + NC group, lots of fibrotic areas and myocardial cell necrosis, interstitial edema were observed, and the surrounding myocardial cells were hypertrophic and disordered. Compared with the MI + NC group, the cardiomyocyte damage in the MI + agomiR-431 group was remarkably alleviated (Figure 3A). In addition, compared with the sham group, serum LDH content and myocardial Caspase-3 activity were notably increased in the MI + NC group. Compared with the MI + NC group, the serum LDH and myocardial Caspase-3 activity were dramatically decreased in the MI + agomiR-431 group (Figure 3B and 3C). On the 7th day after the operation, cardiac function of rats was tested. MI significantly reduced the cardiac function of rats, which was manifested in the decrease of EF and FS. However, myocardial overexpression of miR-431 greatly improved the cardiac function of MI rats (Figure 3D and 3E). From these results, it can be seen that overexpression of miR-431 could reduce myocardial damage and improve cardiac function after MI *in vivo*.

MiR-431 Inhibited Cardiomyocyte Apoptosis by Targeting HIPK3

Through TargetScan software, we found that HIPK3 may have a binding site with miR-431 (Figure 4A). Through Western blot and RT-PCR, we found that H_2O_2 could increase HIPK3 expression, but miR-431 overexpression markedly inhibited that (Figure 4B and 4C). In addition, up-regulation of miR-431 notably inhibited the Luciferase activity in HIPK3-WT + mimic group but failed in HIPK3-MUT + mimic group (Figure 4D). In sum, these results indicated that HIPK3 is a target gene of miR-431.

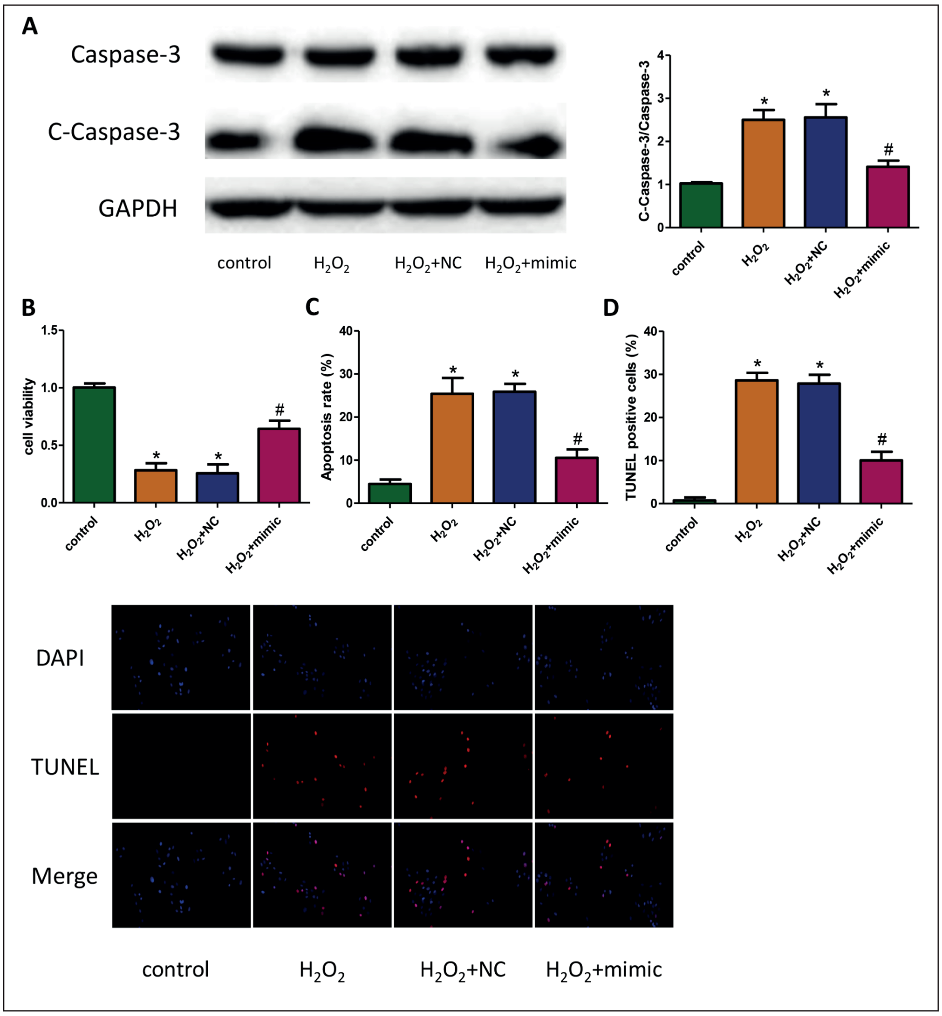

Figure 2. Overexpression of miR-431 inhibited H_2O_2-induced cardiomyocyte apoptosis. **A**, Expression levels of apoptosis-related proteins (Caspase-3 and Cleaved Caspase-3) were detected by RT-qPCR ("*" *p*<0.05 *vs.* control, "#" *p*<0.05 *vs.* H_2O_2+NC, n=3). **B**, CCK-8 assay suggested that miR-431 overexpression restored cell viability ("*" *p*<0.05 *vs.* control, "#" *p*<0.05 *vs.* H_2O_2+NC, n=3). **C**, Overexpression of miR-431 significantly reduced the apoptosis rate ("*" *p*<0.05 *vs.* control, "#" *p*<0.05 *vs.* H_2O_2+NC, n=3). **D**, TUNEL staining showed that miR-431 overexpression can significantly reduce the increase of H9c2 cell apoptosis caused by H_2O_2 (magnification: 200×) ("*" *p*<0.05 *vs.* control, "#" *p*<0.05 *vs.* H_2O_2+NC, n=3).

Discussion

The incidence of AMI increases year by year and has become one of the highest mortality diseases in human beings. During myocardial infarction, ischemia and hypoxia of myocardial cells can cause necrosis and apoptosis of myocardial cells. The central part of the infarct is usually dominated by myocardial necrosis, and the peripheral part of the infarct is dominated by apoptosis. Although thrombolysis and coronary stent implantation can quickly open blood vessels to avoid the continuous expansion of myocardial necrosis area, it is also accompanied by myocardial ischemia-reperfusion injury, which can also cause myocardial cell necrosis and apoptosis[13]. Necrosis and apoptosis of cardiomyocytes cause the number of cardiomyocytes to decrease, which is one of the main reasons for the development of heart failure[14,15].

Thousands of miRNAs have been found to regulate the physiological functions of cells in eukaryotes. MiRNA inhibits the translation of the target gene mRNA or promotes its degradation by complementary base pairing with the 3'UTR of the target gene, thereby participating in the regulation of target protein expression[16]. In recent years, a large amount of evidence shows that miRNAs are involved in the regulation of multiple biological processes of cardiovascular disease, including endothelial cell dysfunction, changes in cell adhesion, platelet growth and rupture, and proliferation and apoptosis of cardiomyocytes[17,18]. Maintaining and restoring the stable expression of miRNA in target organs such as the heart may become a new target for the treatment of cardiovascular diseases. A large amount of data indicates that miRNA not only strictly controls the process of apoptosis in tumor cells, but also participates in

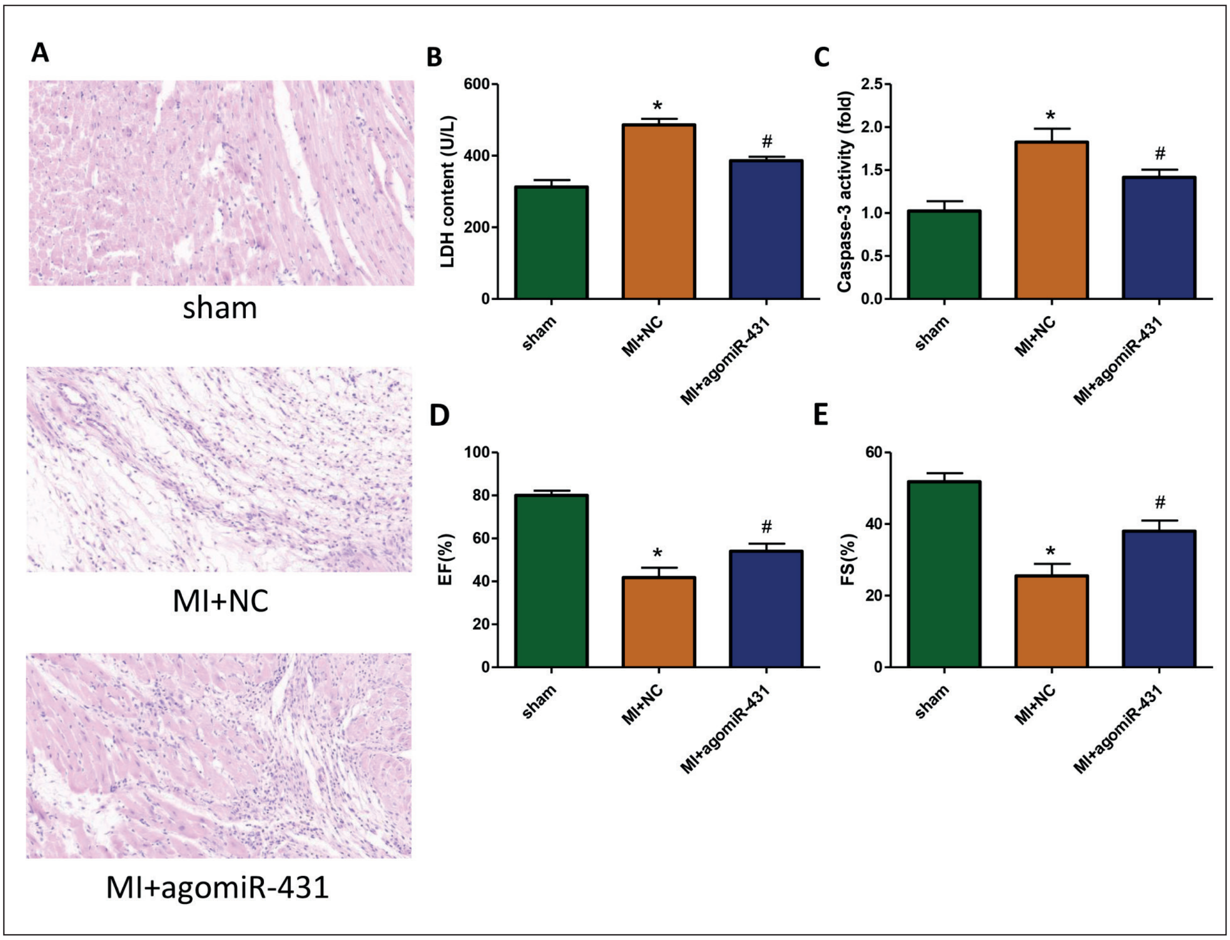

Figure 3. Overexpression of miR-431 improved cardiac function in MI rats. **A**, Typical HE staining of myocardial tissues (magnification: 200×). **B**, Serum LDH contents of rats in 3 groups ("$_*$" p<0.05 *vs.* sham, "$_\#$" p<0.05 *vs.* MI+NC, n=6). **C**, Myocardial Caspase-3 activity of rats in 3 groups ("$_*$" p<0.05 *vs.* sham, "$_\#$" p<0.05 *vs.* MI+NC, n=6). **D, E,** Left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) ("$_*$" p<0.05 *vs.* sham, "$_\#$" p<0.05 *vs.* MI+NC, n=6).

the regulation of apoptosis in cardiomyocytes. Yang et al[19] found that miR-1 expression is increased in ischemic cardiomyocytes, and plays an important role in the regulation of apoptosis, and its expression level plays a decisive role in the degree of heart damage. At the same time, Xu et al[20] showed that miR-1 and miR-133 regulate apoptosis signaling pathways in oxidative stress of rat cardiomyocytes. During oxidative stress, miR-1 expression is markedly increased and miR-1 promotes apoptosis, whereas miR-133, as an anti-apoptotic factor, regulates cardiomyocyte survival[20]. A recent study showed that overexpression of miR-431 attenuates hypoxia/reoxygenation-induced myocardial damage through regulating the expression of ATG3[21].

HIPK3 (homeodomain-interacting protein kinase 3) belongs to the DYRK kinase (dual specificity tyrosine regulated kinase) family in terms of protein structure[22]. Its family members include HIPK1, HIPK2, HIPK3, and HIPK4. By changing the phosphorylation state of target proteins, they participate in biological processes such as cell proliferation, differentiation, and apoptosis[23]. HIPK3 mainly exists in the nucleus and cytoplasm and is widely expressed in human and other mammalian tissue cells and is mainly involved in regulating apoptosis of tumor cells.

In our study, we established a cell model and an animal model of MI, which revealed that the expression of miR-431 decreased in infarcted myocardium. In the next *in vivo* and *in vitro* experiments, we demonstrated that overexpression of miR-431 can greatly inhibit cardiomyocyte apoptosis, thereby reducing cardiomyocyte damage and improving cardiac function. Through the

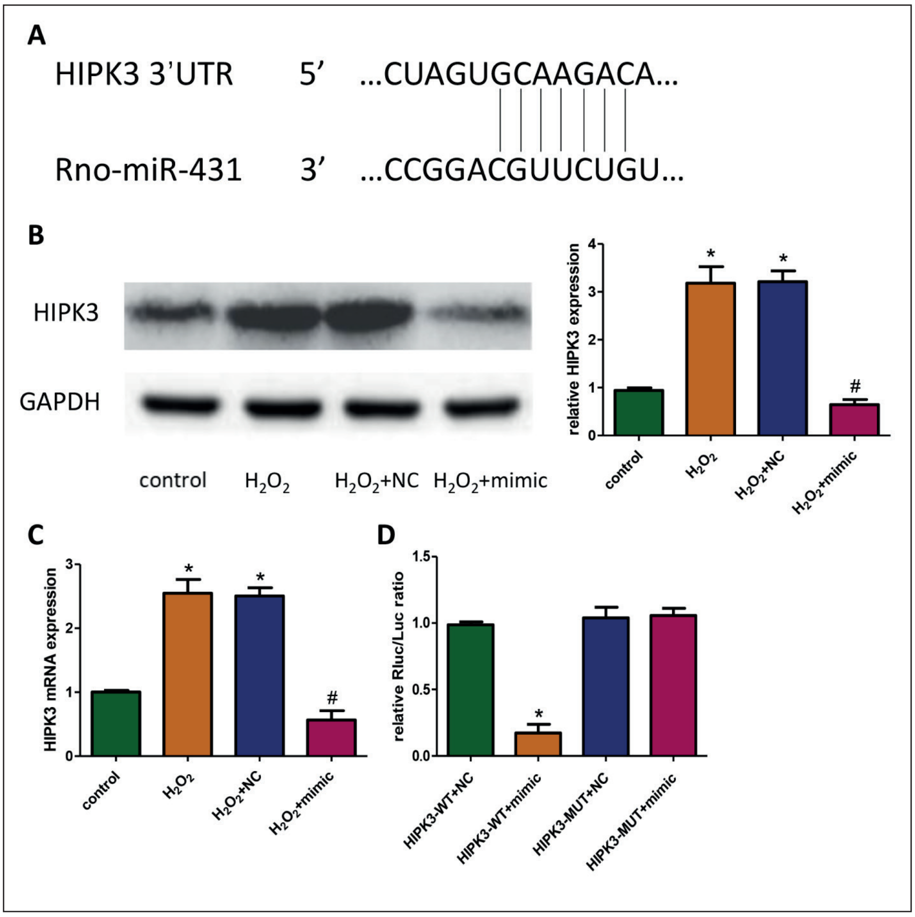

Figure 4. MiR-431 directly targets HIPK3. **A**, Binding site predicted by the TargetScan database. **B**, The expression of HIPK3 was detected using Western blot ("*" p<0.05 *vs.* control, "#" p<0.05 *vs.* H_2O_2+NC, n=3). **C**, The expression of HIPK3 mRNA was detected using RT-PCR ("*" p<0.05 *vs.* control, "#" p<0.05 *vs.* H_2O_2+NC, n=3). **D,** Luciferase activity assay of HEK293T cells transfected with Luciferase constructs containing WT-3′UTR and Mut-3′UTR of HIPK3 ("*" p<0.05 *vs.* HIPK3-WT+NC, n=3).

Luciferase reporter gene experiment, we found that anti-cardiomyocyte apoptosis effect of miR-431 was achieved by targeting HIPK3.

Conclusions

We revealed for the first time that miR-431 expression is reduced in MI. Using miR-431 mimic and miR-431 agomiR to up-regulate the expression of miR-431 in myocardium *in vivo* and *in vitro*, we found that reconstructed miR-431 expression can reduce myocardial cell damage in MI by inhibiting cardiomyocyte apoptosis. Our research may provide a potential target for the treatment of MI. In conclusion, miR-431 expression was decreased in infarcted myocardium and up-regulation of miR-431 could inhibit myocardial apoptosis and improve cardiac function in MI rats through targeting HIPK3.

Conflict of Interests

The authors declare that they have no conflict of interests.

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