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LncRNA MALAT1 knockdown alleviates myocardial apoptosis in rats with myocardial ischemia-reperfusion through activating PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: To observe the effect of long non-coding ribonucleic acid metastasis-associated lung adenocarcinoma transcript 1 (lncRNA MALAT1) on the myocardial ischemia-reperfusion (I/R) injury in rats and to explore its potential mechanism, to provide certain references for clinical prevention and treatment of myocardial I/R injury.

MATERIALS AND METHODS: A total of 60 male Wistar rats were randomly divided into the Control group (n=20), I/R group (n=20) and I/R + MALAT1 small-interfering RNA (siRNA) group (n=20) using a random number table. The I/R model was established through recanalization after ligation of left anterior descending coronary artery (LAD), and the MALAT1 knockdown model was established via tail intravenous injection of MALAT1 siRNA in the I/R + MALAT1 siRNA group. The ejection fraction (EF%) and fractional shortening (FS%) of rats in each group were detected via echocardiography and the infarction area in each group was detected using 2,3,5-triphenyl tetrazolium chloride (TTC) assay. Moreover, the morphological changes in myocardial cells in each group were detected via hematoxylin-eosin (H&E) staining, and the myocardial apoptosis level was detected via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. At the same time, the expression levels of the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) and pro-apoptotic protein Bcl-2 associated X protein (Bax) in myocardial tissues in each group were determined via Western blotting. Finally, the effect of MALAT1 knockdown on the phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) protein expression was detected via Western blotting.

RESULTS: The expression level of lncRNA MALAT1 in myocardial tissues was significantly higher in the I/R group than that in the Control group ($p<0.05$). The MALAT1 knockdown could significantly improve the cardiac insufficiency caused by I/R injury, and increase both EF% and FS% in rats ($p<0.05$). In addition, the MALAT1 knockdown could markedly inhibit myocardial infarction caused by I/R injury and reduce the infarction area from (62.12 ± 1.29) to (27.66 ± 3.58; $p<0.05$). The results of the H&E staining showed that the myofilaments were arranged more orderly, the degrees of degradation and necrosis were lower and the cellular edema was significantly alleviated in the I/R + MALAT1 siRNA group compared with those in the I/R group. According to the results of TUNEL staining, the rats in I/R + MALAT1 siRNA group had a markedly lower level of myocardial apoptosis than the I/R group ($p<0.05$), and the Bax/Bcl-2 ratio also remarkably declined in the I/R + MALAT1 siRNA group ($p<0.05$). Furthermore, the results of Western blotting revealed that MALAT1 siRNA could significantly reverse the I/R injury-induced inhibition on the AKT phosphorylation ($p<0.05$).

CONCLUSIONS: The MALAT1 knockdown can markedly improve the I/R-induced myocardial injury and promote the cardiac function of rats, whose mechanism may be related to the activation of the AKT signaling pathway by MALAT1 siRNA. Therefore, lncRNA MALAT1 is expected to be a new therapeutic target for myocardial I/R injury.

Key Words:
MALAT1, Myocardial ischemia-reperfusion, Myocardial cells, Apoptosis, PI3K/AKT.

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Introduction

Myocardial infarction (MI) is the major cause of death and an important public health problem in the world[1,2]. The early percutaneous coronary intervention (PCI) is the most effective treatment means for MI currently[3]. However, the continuous reperfusion after ischemia usually causes secondary damage to the myocardium, which is known as myocardial ischemia-reperfusion (I/R) injury[4]. I/R injury is an inevitable pathophysiological phenomenon in the treatment of ischemic heart disease and open heart operation, which can lead to reperfusion arrhythmia, transient mechanical dysfunction, myocardial stunning and other pathological changes[5]. Therefore, inhibiting myocardial I/R injury is of great significance in the prevention and treatment of ischemic cardiomyopathy, especially MI.

Long non-coding ribonucleic acids (lncRNAs) are a kind of long-chain RNA molecules with more than 200 nucleotides in the transcription length[6]. Although lncRNAs cannot encode the corresponding proteins in cells, they can regulate the expression of the corresponding target genes at the transcriptional/post-transcriptional level and epigenetic modification, ultimately affecting the occurrence and development of diseases[7,8]. As a member of the lncRNA family, lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) plays an important role in a variety of diseases, including tumors, cardiovascular diseases and endocrine diseases[9]. For example, lncRNA MALAT1, a competitive endogenous RNA, regulates the expression of ZEB2 in clear cell renal carcinoma[10] through the sponge adsorption of micro RNA (miR)-200s. In the rat model of diabetic cardiomyopathy, inhibiting lncRNA MALAT1[11] can reduce the myocardial apoptosis and promote the cardiac function. In addition, lncRNA MALAT1[12] up-regulates Smad4 *via* the sponge adsorption of miR-204, ultimately facilitating the osteogenic differentiation of human aortic valve interstitial cells. However, there have been no reports yet on the role of lncRNA MALAT1 in myocardial I/R injury.

In the present study, the myocardial I/R model was established using lncRNA MALAT1 knockdown to detect the effects of MALAT1 knockdown on the cardiac function, myocardial apoptosis and infarction area in rats and analyze the potential molecular mechanism of MALAT1 in affecting myocardial I/R injury.

Materials and Methods

Laboratory Animal Grouping and Modeling

A total of 60 male Wistar rats aged 12-14 weeks and weighing (288.31 ± 10.52) g were randomly divided into the Control group (n=20), I/R group (n=20) and I/R + MALAT1 small-interfering RNA (siRNA) group (n=20) using a random number table. This study was approved by the Animal Ethics Committee of Capital Medical University Animal Center. There were no statistically significant differences in such basic data as week age and body weight among the three groups. MALAT1 siRNA at a certain dose (4 mmol/kg) was injected into rats *via* the tail vein in I/R + MALAT1 siRNA group. The I/R model was established as follows: the rats in each group were anesthetized *via* intraperitoneal injection of pentobarbital sodium (50 mg/kg), and the cannula was inserted into the left carotid artery to measure the blood pressure of rats. The heart rate was monitored using the two-lead electrocardiograph (ECG). Then, the thorax was cut open in the 4th intercostal space and the pericardium was excised to expose the heart. The left anterior descending coronary artery (LAD) was ligated at 2 mm above the left auricle using the 6-0 silk thread to induce local myocardial ischemia. At 30 min after ischemia, the silk thread was loosened, followed by reperfusion for 2 h. In the Control group, the rats underwent the same operation, but LAD was not ligated using the silk thread. After reperfusion, the rats were executed, and the myocardial tissues of the left ventricular anterior wall were extracted. After the blood residue was washed away with normal saline, the tissues were placed in a refrigerator at -80°C for later use (Figure 1).

Echocardiography

To detect the cardiac function of rats in each group, echocardiography was performed using the Mylab 30CV ultrasound system (Esaote S.P.A., Genoa, Italy) and 10-MHz linear ultrasound transducer. Then, the chest hair was shaved off and the rats were anesthetized and placed on a heating plate at 37°C with the left side upwards. Finally, ejection fraction (EF%), fractional shortening and heart rate (bpm) were detected.

1.3 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

1) The fresh myocardial tissues were placed into the grinding tool of heart sections and fro-

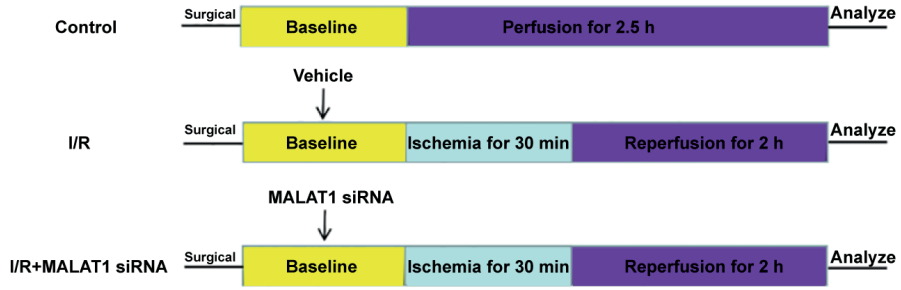

Figure 1. Treatment flow chart of rats in each group. Control: Control group, I/R: ischemia-reperfusion group, I/R+MALAT1 siRNA: ischemia-reperfusion + MALAT1 knockdown group.

zen in the refrigerator at -20°C for 30 min. 2) The myocardial tissues were sliced into 2 mm-thick sections (not more than 6 sections for each tissue). 3) The sections were placed in fresh TTC (Beyotime, Shanghai, China) solution (2%) for incubation for not less than 0.5 h. 4) After 0.5 h, the sections were taken out, fixed with 4% paraformaldehyde (Beyotime, Shanghai, China) and photographed.

Hematoxylin-Eosin (H&E) Staining

The heart obtained in each group was placed in 10% formalin overnight, dehydrated and embedded in paraffin. Then, all myocardial tissues were sliced into 5 μm-thick sections, fixed on a glass slide and baked dry, followed by staining. According to the instructions, the sections were soaked in xylene, ethanol in gradient concentration and hematoxylin, respectively, and sealed with resin. After drying, the sections were observed and photographed under a light microscope to observe the morphology of myocardial cells, cardiac interstitium and myofilament.

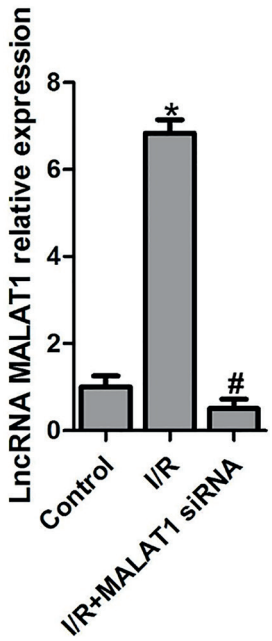

Figure 2. Expression of lncRNA MALAT1 in myocardial tissues in I/R injury rats. Control: Control group, I/R: ischemia-reperfusion group, I/R+MALAT1 siRNA: ischemia-reperfusion + MALAT1 knockdown group. The difference is statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL)

The myocardial tissue sections were baked in an oven at 60°C for 30 min, deparaffinized with xylene (5 min/3 times), and dehydrated with 100% ethanol, 95% ethanol and 70% ethanol 3 times each. Then, the sections were incubated with protein kinase K for 0.5 h, washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), reacted with the terminal deoxynucleotidyl transferase (TdT) and Luciferase-labeled dUTP (Novus Biologicals, Littleton, CO, USA) at 37°C for 1 h, and incubated again with the horseradish peroxidase (HRP)-labeled specific antibody in an incubator at 37°C for 1 h, followed by reaction with diaminobenzidine (DAB) as the substrate at room temperature for 10 min. The nucleus was stained with hematoxylin, followed by photography and counting under the light microscope.

Detection of Expression of Related Genes via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) The total RNA was extracted from myocardial tissues using the TRIzol method (Invitrogen, Carlsbad, CA, USA), the concentration and purity of RNA extracted were detected using an ultravi-

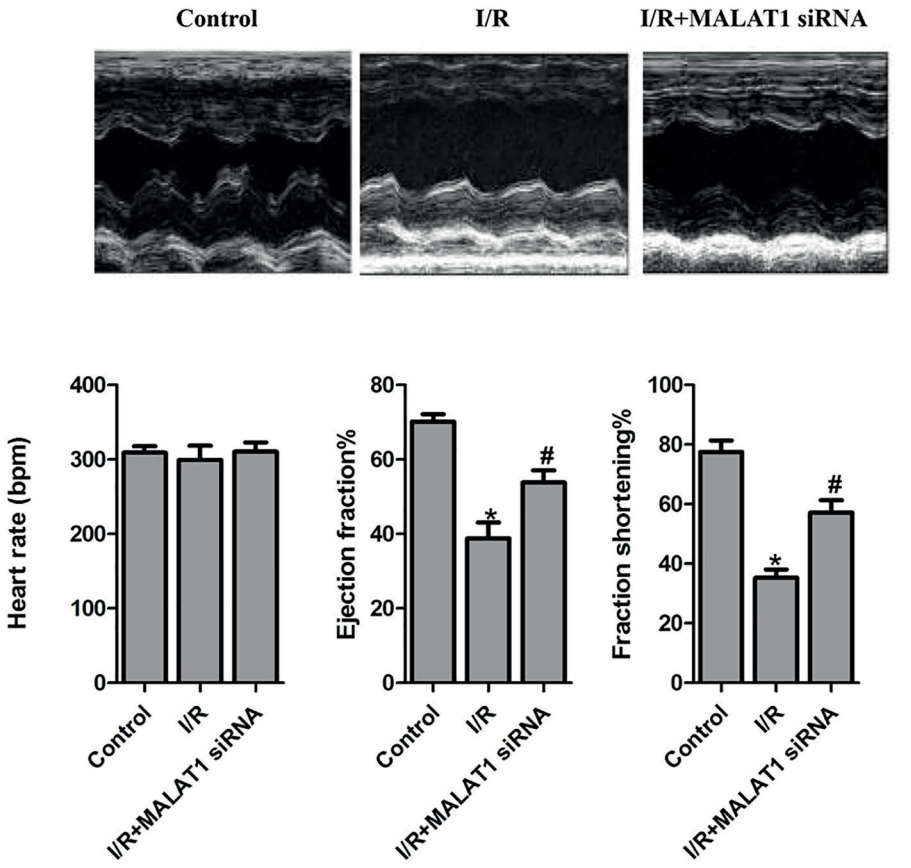

Figure 3. Effect of MALAT1 knockdown on cardiac function of rats in each group. Control: Control group, I/R: ischemia-reperfusion group, I/R+MALAT1 siRNA: ischemia-reperfusion + MALAT1 knockdown group. The difference is statistically significant * *vs*. Control group and # *vs*. I/R group ($p<0.05$).

olet spectrophotometer, and the RNA with absorbance (A) $_{260}/A_{280}$ of 1.8-2.0 could be used. (2) The messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in the refrigerator at -80°C. (3) Reverse Transcription-Polymerase Chain Reaction (RT-PCR) system: 2.5 μL 10 × Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2×10^6 U/L) and 19 μL ddH_2O. The amplification system of RT-PCR was the same as above. The gene primer sequences are shown in Table I.

Western Blotting

The myocardial tissues of rats in each group were fully ground in the lysis buffer, followed by ultrasonic lysis. Then, lysis buffer was centrifuged and the supernatant was taken and placed into an Eppendorf (EP; Hamburg, Germany) tube. The protein concentration was detected *via* ultraviolet spectrometry, and the protein samples were

Table I. Primer sequences for RT-PCR.

Target gene		Primer sequence
GAPDH	Forward	5'-GACATGCCGCCTGGAGAAAC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGT-3'
MALAT1	Forward	5'-TGCTGCCTTTTCTGTTCCTT-3'
	Reverse	5'-AAGGTGCTGGGTAGGGAAGT-3'

quantified to be the same concentration. Subsequently, the protein was sub-packaged and placed in the refrigerator at -80°C. After the total protein was extracted, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Then, protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), incubated with the primary antibody at 4°C overnight, and then incubated again with the goat anti-rabbit secondary antibody (Abcam, Cambridge, MA, USA) in a dark place for 1 h. The protein band was scanned and quantified using the Odyssey scanner, and the level of the protein to be detected was corrected using glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (SPSS Inc., Chicago, IL, USA) software was used for the analysis of all data. Measurement data were expressed as mean ± standard deviation, and *t*-test was used for the comparison of data between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of LncRNA MALAT1 in Myocardial Tissues in I/R Injury Rats

The expression level of lncRNA MALAT1 in myocardial tissues was detected in the Control group, I/R group and I/R + MALAT1 siRNA group. As shown in Figure 2, the expression level of lncRNA MALAT1 in myocardial tissues was significantly increased in the I/R group ($p<0.05$). After MALAT1 siRNA was injected *via* the tail vein, the expression level of lncRNA MALAT1 in myocardial tissues was markedly inhibited ($p<0.05$), indicating that the rat model of MALAT1 knockdown was successfully induced.

Effect of MALAT1 Knockdown on Cardiac Function of Rats in Each Group

As shown in Figure 3, the results of echocardiography displayed that there was no statistically significant difference in the heart rate among the three groups, so the influences of heart rate on EF% and FS% in each group could be eliminated. Compared with those in the Control group, the ventricular cavity was enlarged and the heart wall became thinner in the I/R group. The abnormal changes in the heart structure induced by I/R could be significantly ameliorated after MALAT1 knockdown. Furthermore, FS% and EF% were detected in each group, and it was found that MALAT1 knockdown significantly reversed the decline in them in the I/R group ($p<0.05$), suggesting that suppressing lncRNA MALAT1 can improve the cardiac function of I/R injury rats.

Effect of MALAT1 Knockdown on Infarction Area in I/R Injury Rats

The infarction area in each group was evaluated *via* TTC staining. The results showed that the infarction area was 0.53 ±0.51 *vs*. 39.14 ± 2.56 *vs*. 19.25±1.82, respectively, in the three groups, and there were statistically significant differences ($p<0.05$; Figure 4), suggesting that inhibiting lncRNA MALAT1 can effectively reduce the infarction area in I/R injury rats.

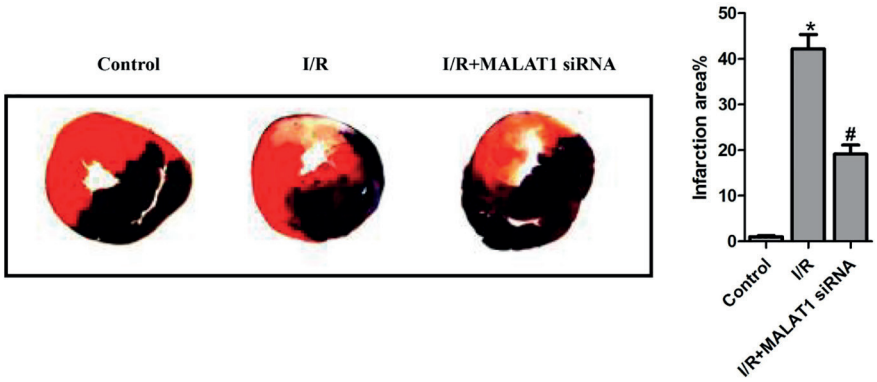

Figure 4. Effect of MALAT1 knockdown on infarction area in each group. Control: Control group, I/R: ischemia-reperfusion group, I/R+MALAT1 siRNA: ischemia-reperfusion + MALAT1 knockdown group. The difference is statistically significant * *vs*. Control group and # *vs*. I/R group ($p<0.05$).

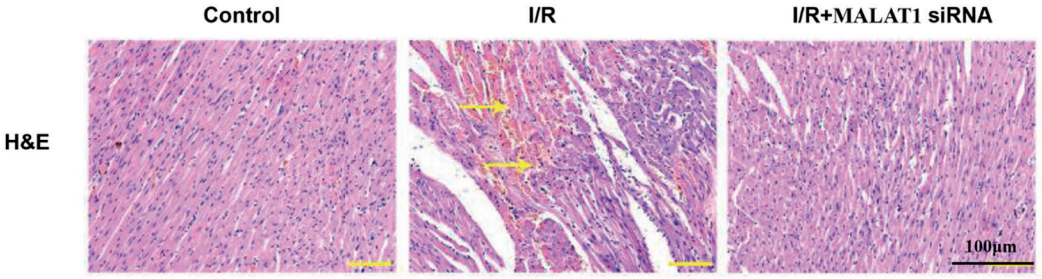

Figure 5. H&E staining results in each group (magnification: 100×). Control: Control group, I/R: ischemia-reperfusion group, I/R+MALAT1 siRNA: ischemia-reperfusion + MALAT1 knockdown group.

H&E Staining Results in Each Group

To evaluate the microstructural changes in myocardial cells in the cross-section of the heart, H&E staining was performed for the myocardial tissues. In the I/R group, there was significant edema in myocardial cells, the myofilaments were arranged disorderly, and varying degrees of degradation and necrosis occurred, accompanied by inflammatory cell infiltration. After MALAT1 knockdown, myocardial tissue edema was markedly alleviated and the abnormalities in myofilaments were also remarkably ameliorated (Figure 5), indicating that MALAT1 knockdown can alleviate the myocardial I/R injury.

Effect of MALAT1 Knockdown on Myocardial Apoptosis

The myocardial apoptosis level was detected *via* TUNEL staining in the three groups. The results manifested that after I/R injury, the apoptosis of myocardial cells and fibroblasts in myocardial tissues was remarkably increased ($p<0.05$), about (39.15 ± 1.89) times that in the Control group. After MALAT1 knockdown, the number of apoptotic myocardial cells declined to (21.45 ± 2.66) times that in the Control group ($p<0.05$; Figure 6), indicating that MALAT1 knockdown can significantly suppress myocardial apoptosis.

Effect of MALAT1 Knockdown on Apoptosis-Related Genes in Myocardial Cells

Furthermore, the expression levels of apoptosis-related genes Bax and Bcl-2 in the myocardium were detected in each group. The results showed that the Bax/Bcl-2 ratio was markedly increased in the I/R group, indicating the increased level of myocardial apoptosis. Compared with

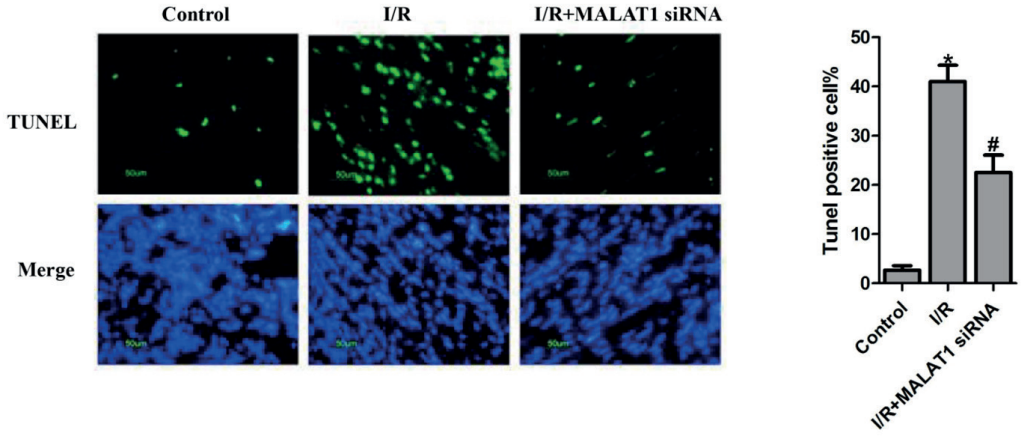

Figure 6. Effect of MALAT1 knockdown on myocardial apoptosis (magnification: 40×). Control: Control group, I/R: ischemia-reperfusion group, I/R+MALAT1 siRNA: ischemia-reperfusion + MALAT1 knockdown group. The difference is statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

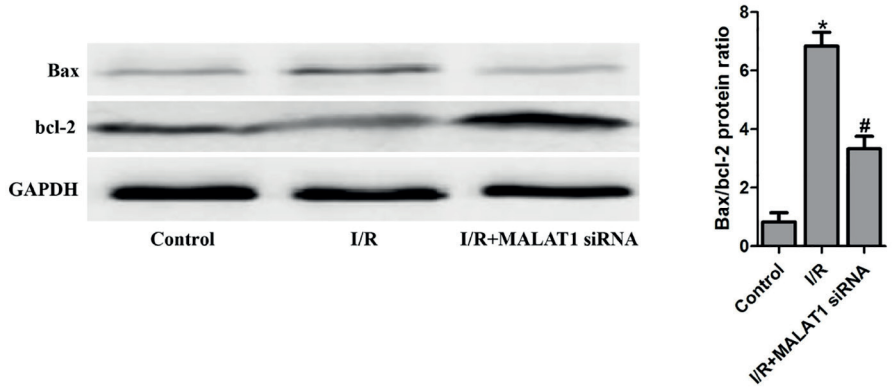

Figure 7. Effect of MALAT1 knockdown on apoptosis-related genes in myocardial cells. Control: Control group, I/R: ischemia-reperfusion group, I/R+MALAT1 siRNA: ischemia-reperfusion + MALAT1 knockdown group. The difference is statistically significant * *vs*. Control group and # *vs*. I/R group ($p<0.05$).

the I/R group, the I/R + MALAT1 siRNA group had a remarkably reduced Bax/Bcl-2 ratio (Figure 7), further confirming the inhibitory effect of MALAT1 siRNA on I/R injury-induced myocardial apoptosis.

Effect of MALAT1 Knockdown on the PI3K/AKT Signaling Pathway in Rats

To deeply explore the effect of MALAT1 knockdown on myocardial I/R injury in rats, the protein expression levels of total protein kinase B (t-AKT) and phosphorylated AKT (p-AKT) in the myocardium were determined *via* Western blotting in the three groups. It can be seen from Figure 8 that MALAT1 knockdown could markedly reverse the I/R injury-induced inhibition on the AKT phosphorylation ($p<0.05$). Therefore, it is speculated that the cardioprotective effect of MALAT1 siRNA may be associated with the activation of the phosphatidylinositol 3-hydroxy kinase (PI3K)/AKT signaling pathway.

Discussion

Acute MI is still one of the major causes of death around the world[13]. The rapid recovery of blood flow through the occluded coronary artery using drug intervention (thrombolysis) or emergency PCI is the most effective strategy to limit the MI

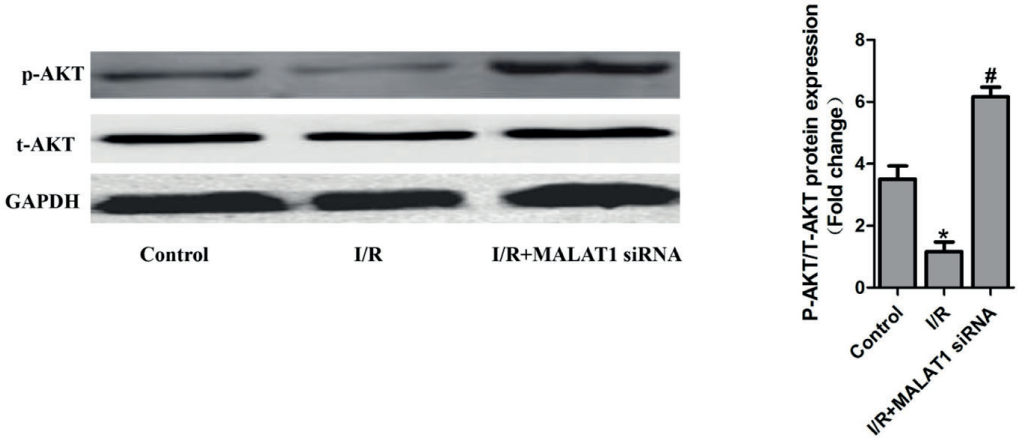

Figure 8. Effect of MALAT1 knockdown on the PI3K/AKT signaling pathway in rats. Control: Control group, I/R: ischemia-reperfusion group, I/R+MALAT1 siRNA: ischemia-reperfusion + MALAT1 knockdown group. The difference is statistically significant * *vs*. Control group and # *vs*. I/R group ($p<0.05$).

area and improve the prognosis of patients with acute MI[14,15]. However, cardiac reperfusion itself will also lead to additional myocardial cell death and further expand the infarction area. The factors leading to reperfusion injury mainly include oxidative stress, inflammation and apoptosis[16]. During myocardial ischemia[17], the massive consumption of adenosine triphosphate in myocardial cells reduces the ability of the sarcoplasmic reticulum to take in Ca^{2+}, resulting in the massive accumulation of mitochondrial Ca^{2+}. During reperfusion, oxygen re-enters myocardial cells, causing damage to mitochondrial electron transport chain and increasing the production of reactive oxygen species (ROS)[18]. The opening of mitochondrial permeability transition pore is promoted by the mitochondrial Ca^{2+} overload and increased the production of ROS, causing cellular energy disorders and ultimately leading to irreversible cell necrosis and apoptosis[19]. Therefore, suppressing myocardial apoptosis, inflammation and oxidative stress during reperfusion can effectively ameliorate I/R injury-induced cardiac dysfunction and reduce infarction area.

LncRNA MALAT1 is one of the most abundant and highly-conserved lncRNAs in mammals, which, unlike most other lncRNAs, displays the rare 3'-end processing mechanism. In addition, its specific nuclear localization, developmental regulation and abnormal expression in tumor tissues indicate its key biological functions in human growth and development, and occurrence and development of various diseases[20]. For example, the abnormal expression of lncRNA MALAT1 is closely associated with diabetes-associated microangiopathies, such as diabetic retinopathy. In the retinal vascular endothelial cells of streptozotocin-induced diabetes rats and db/db diabetes mice, the expression level of lncRNA MALAT1 is significantly up-regulated, while inhibiting lncRNA MALAT1 can markedly alleviate the loss of pericytes, capillary degeneration, microvascular leakage and retinal inflammation in rats/mice. Additionally, the down-regulation of lncRNA MALAT1 can regulate the proliferation, migration and angiogenesis of retinal endothelial cells *in vitro*. According to further studies[21], the regulatory effect of lncRNA MALAT1 on high glucose-induced endothelial cell dysfunction is associated with the p38 MAPK signaling pathway. In septic cardiomyopathy, lncRNA MALAT1 suppresses the p38 MAPK/NF-κB signaling pathway[22] through the interaction with miR-125b, ultimately alleviating myocardial inflammation. Moreover, it has been reported in the literature that MALAT1 is highly expressed in patients with acute MI[23]. Autophagy is an intracellular protein or metabolite degradation process that plays an important role in keeping cellular homeostasis, but the excessive activation of autophagy can lead to self-digestion and cell death, so it has a key role in various heart diseases, such as myocardial I/R injury. MiR-204 protects myocardial cells from I/R injury by inhibiting autophagy, and lncRNA can act as the endogenous miRNA sponge to interact with miRNAs and regulate their expression. Moreover, there are reports that lncRNA MALAT1 contains the binding sites for miR-204, so it can serve as the endogenous sponge inhibiting miR-204[24]. In the present study, it was found that inhibiting lncRNA MALAT1 could significantly improve the cardiac dysfunction caused by I/R injury, reduce the infarction area in myocardial tissues, lower the number of apoptotic myocardial cells and alleviate the pathological injury of myocardial tissues. Further studies showed that the PI3K/AKT signaling pathway in myocardial tissues was markedly activated after MALAT1 knockdown. However, there are still some deficiencies in the present report: 1) The cell experiment was not designed for verification, and 2) whether the regulatory effect of lncRNA MALAT1 on myocardial apoptosis depends on the activation of the AKT signaling pathway was not verified.

Conclusions

It was found, for the first time, that lncRNA MALAT1 knockdown can alleviate the I/R injury-induced myocardial apoptosis in rats, whose mechanism may be associated with the regulatory effect of lncRNA MALAT1 on AKT.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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