Effects of lncRNA MALAT1-mediated β-catenin signaling pathway on myocardial cell apoptosis in rats with myocardial ischemia/reperfusion injury

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

MATERIALS AND METHODS: A total of 60 male Sprague-Dawley rats were divided into 3 groups using a random number table, including the Sham group (n=20), I/R group (n=20) and I/R + MALAT1 small interfering RNA (siRNA) group (n=20). An I/R model was established by means of recanalization after ligation of the left anterior descending coronary artery of the rats. The rats in the I/R + MALAT1 siRNA group were used to establish a model of MALAT1 knockdown by injecting MALAT1 siRNA from the tail vein. The myocardial infarction area in each group was detected via 2,3,5-triphenyl tetrazolium chloride (TTC) staining. The ejection fraction% (EF%) and fractional shortening% (FS%) of the heart in each group were measured through echocardiography. Hematoxylin and eosin (H&E) staining was adopted to determine the morphological changes in myocardial cells in each group. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to detect the apoptosis levels of myocardial cells and fibroblasts in the cardiac tissues in each group, and Western blotting assay was conducted to measure the expression levels of apoptosis-related proteins [B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax)]. In addition, the content of β-catenin in the three groups of rats was determined via immunohistochemical staining. Finally, the impacts of MALAT1 siRNA on the expression level of β-catenin protein were detected using Western blotting assay.

RESULTS: MALAT1 siRNA could prominently ameliorate the I/R-induced cardiac insufficiency in the rats and improve the EF% and FS% of the heart ($p<0.05$). Moreover, MALAT1 siRNA was able to remarkably inhibit the I/R injury-induced myocardial infarction, reducing the infarction area from (59.54±3.45) to (24.85±1.30; $p<0.05$). The results of the H&E staining indicated that compared with those in the I/R group, the myofilaments of the myocardial cells were well-arranged, the degrees of degradation and necrosis of the myofilaments declined, and the cellular edema was relieved markedly in the I/R + MALAT1 siRNA group. It was shown in the results of immunohistochemistry and Western blotting that MALAT1 siRNA could notably reverse the I/R-induced up-regulation of β-catenin expression ($p<0.05$).

CONCLUSIONS: MALAT1 knockdown can significantly ameliorate the I/R-induced myocardial injury and improve the cardiac function of the rats, whose mechanism is probably correlated with the inhibition of MALAT1 siRNA on β-catenin. Therefore, MALAT1 siRNA is expected to become a new target for the treatment of myocardial infarction.

Key Words:
  MALAT1, Myocardial ischemia/reperfusion, Myocardial cells, Apoptosis, β-catenin.

Introduction

Ischemic heart disease is not only a leading cause of death but also a major public health prob-

lem around the world[1]. Reperfusion is an important strategy for the heart to remedy irreversible damage to the myocardial tissues, while the postischemic reperfusion usually causes a certain degree of injury to the myocardium, which is known as ischemia/reperfusion (I/R) injury[2]. As an inevitable pathophysiological phenomenon in patients during the treatment of ischemic heart disease and cardiac thoracic surgery, the I/R injury can result in reperfusion arrhythmia, transient mechanical dysfunction, myocardial stunning and other pathological changes[3,4]. Hence, repressing the myocardial I/R injury is of great significance for the prevention and treatment of ischemic cardiomyopathy. Studies[5,6] have manifested that the myocardial I/R injury has close correlations with various factors, such as massive production of oxygen free radicals, change in cardiac hemodynamics, calcium overload in myocardial cells, inflammation as well as necrosis and apoptosis of myocardial cells. Furthermore, large quantities of animal experiments and clinical studies have verified that the abnormal inhibition on the β-catenin signaling pathway is a vital cause of I/R injury[7]. Therefore, taking β-catenin as the therapeutic target is the key strategy of repressing the myocardial I/R injury.

Long non-coding ribonucleic acids (lncRNAs) refer to long RNA molecules with a transcript length of over 200 nucleotides[8]. LncRNAs themselves cannot encode corresponding proteins in cells, but they can regulate the expression of corresponding target genes through (post-) transcriptional level, epigenetic modification and other levels, ultimately affecting the occurrence and development of diseases[9,10]. LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), a member of the lncRNA family, plays crucial roles in various diseases, including tumors, cardiovascular diseases and endocrine disorders[11]. For example, as a type of competitive endogenous RNA, lncRNA MALAT1 can regulate ZEB2 expression in clear cell renal carcinoma by sponging microRNA (miR)-200s[12]. However, the role of lncRNA MALAT1 in the myocardial I/R injury has not been reported yet.

In this research, a rat model of MALAT1 knockdown was established through tail vein injection of MALAT1 small interfering RNA (siRNA), and a rat model of myocardial I/R injury was established by ligating the coronary artery at the same time, to investigate the action and mechanism of lncRNA MALAT1 in the occurrence and development of myocardial I/R injury.

Materials and Methods

Grouping and Treatment of Laboratory Animals

This study was approved by the Animal Ethics Committee of the Guangxi Medical University Animal Center. A total of 60 male Sprague-Dawley rats aged 12-14 weeks and weighing (285.61±10.66) g were divided into the Sham group (*n*=20), I/R group (*n*=20) and I/R + MALAT1 siRNA group (*n*=20) using a random number table. There were no statistical differences in the week age, weight and other basic information among the three groups of rats. The rats in the I/R + MALAT1 siRNA group were injected with MALAT1 siRNA (2 mL/kg/d) *via* the tail vein. The specific surgical procedures are as follows: the rats in each group were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital, and then the cannula was inserted into the left carotid artery to detect the blood pressure of the rats. The ECG using bipolar limb leads were applied to measure the heart rate. The thoracic cage was opened from the fourth intercostal space, and the pericardium was excised to expose the heart. Next, the left anterior descending coronary artery was ligated at 2 mm above the left atrial appendage using a 6-0 silk suture to induce local myocardial ischemia. 30 min later, the silk suture was released for reperfusion for 2 h. The rats in the Sham group were subjected to the same surgical procedures except for the ligation with silk suture. After reperfusion, the rats were sacrificed to acquire the myocardial tissues on the left ventricular anterior wall, and the tissues were placed into a refrigerator at -80°C for standby use after the blood was rinsed with normal saline (Figure 1).

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

(1) The total RNA in the myocardial tissues was extracted using the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA), whose concentration and purity were measured using an ultraviolet spectrophotometer, and the RNA with A_{260}/A_{280} =1.8-2.0 was eligible for use. (2) The mRNA was synthesized into complementary deoxyribonucleic acid (cDNA) through RT, and the cDNA was 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
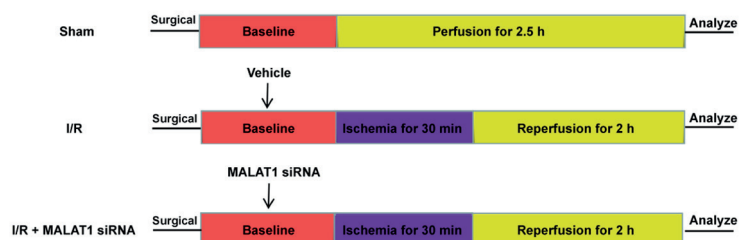
Figure 1. Process flow diagram for each group of rats. Sham: Sham group, I/R: I/R group, I/R + MALAT1 siRNA: I/R + MALAT1 knockdown group.

mmol/L), 0.5 μL Taq polymerase (2×10^6 U/L) and 19 μL ddH$_2$O. The RT-PCR amplification system was the same. The primer sequences for genes applied in this research are shown in Table I.

Detection Via Echocardiography

To determine the cardiac functions of the rats in each group, MyLab 30CV ultrasound system (Esaote S.p.A, Genoa, Italy) and a 10-MHz linear ultrasonic transducer were used to obtain the echocardiography. After the hair in the anterior thoracic region was shaved off, the rats were anesthetized and put on a heating plate at 37°C, with the left side facing upward. Parameters including ejection fraction% (EF%), fractional shortening% (FS%) and heart rate were measured.

2,3,5-Triphenyl Tetrazolium Chloride (TTC) Staining

1) Fresh myocardial tissues were put into a rat heart grinder and frozen in the refrigerator at -20°C for 30 min for slicing. 2) The myocardial tissues were sliced to about 2 mm-thick sections, with no more than 6 sections in each tissue. 3) The sliced sections were placed in and sufficiently contacted with the freshly-prepared TTC solution (2%), followed by incubation for at least 0.5 h. 4) After that, the sections were taken out and fixed with 4% paraformaldehyde, followed by photography.

Hematoxylin and Eosin (H&E) Staining

The hearts obtained from each group were placed in 10% formalin overnight, followed by dehydration and embedding in paraffin blocks. Subsequently, all the myocardial tissues were sliced to 5 μm-thick sections, fixed on glass slides and dried by baking, and then the sections could be utilized for staining. According to the instructions, the tissues were soaked in xylene, gradient concentrations of ethanol and hematoxylin, and mounted in resin. After air drying, the tissues were observed and photographed under a light microscope. The morphology of the myocardial cells, myocardial interstitium and myofilaments was observed.

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

The sliced 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%, 95% and 70% ethanol 3 times, respectively. Later, the sections were incubated with protein kinase K for half an hour. After rinsing with Phosphate-Buffered Saline (PBS, Gibco, Grand Island, NY, USA) TdT and Luciferase-labeled dUTP were added for reaction at 37°C for 1 h. Next, horseradish peroxidase (HRP)-labeled specific antibodies were added and incubated

Table I. Primer sequences of indexes 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'
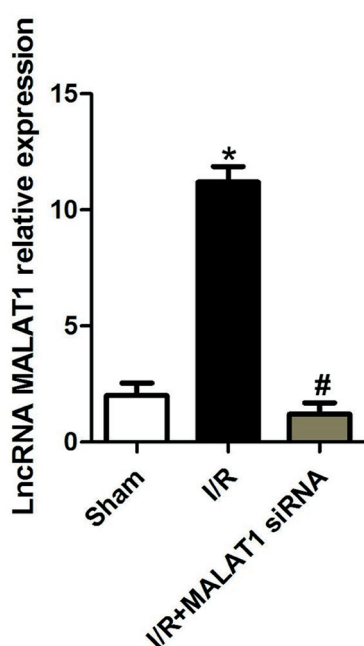
Figure 2. Expression level of lncRNA MALAT1 in the myocardial tissues of the three groups of rats. Sham: Sham group, I/R: I/R group, I/R + MALAT1 siRNA: I/R + MALAT1 knockdown group, *$p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* I/R group, with statistical differences.

again in an incubator at 37°C for 1 h. Then, the sections were reacted at room temperature for 10 min with diaminobenzidine (DAB; Solarbio, Beijing, China) as the substrate, followed by nuclear staining with hematoxylin and photography and counting under a fluorescence microscope.

Western Blotting Assay

The cardiac tissues of each group of rats were fully ground in lysis buffer, followed by ultrasonic lysis. Subsequently, the lysis buffer was centrifuged, and the supernatant was absorbed and subpackaged into Eppendorf tubes (EP; Hamburg, Germany). Later, the protein concentration was determined through the bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA) and ultraviolet spectrophotometric assay, and the volume of all the sample proteins was maintained at equal concentration. Next, the proteins were subpackaged and preserved in the refrigerator at -80°C. The total protein was extracted and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the proteins in the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), followed by incubation with primary antibody at 4°C overnight, incubation with

goat anti-rabbit secondary antibody in the dark for 1 h and scanning and quantification of protein bands using an Odyssey membrane scanner. The level of the proteins to be detected was corrected *via* glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

All the data were analyzed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA), the measurement data were presented as mean ± standard deviation, and the *t*-test was performed for comparison of data between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression Level of LncRNA MALAT1 in the Myocardial Tissues of the Three Groups of Rats

First, the expression level of lncRNA MALAT1 in the heart of the three groups of rats was detected *via* RT-PCR. It was shown that the expression level of lncRNA MALAT1 in the myocardial tissues in the I/R group was elevated remarkably compared with that in the Sham group ($p<0.05$). However, the expression of lncRNA MALAT1 in the myocardial tissues of the rats was suppressed markedly after the injection of MALAT1 siRNA ($p<0.05$), proving that the rat model of lncRNA MALAT1 knockdown is induced successfully (Figure 2).

Effects of LncRNA MALAT1 on the Cardiac Function of the Rats with Myocardial I/R Injury

According to the results of echocardiography (Figure 3), there was no statistical difference in the heart rate among the three groups of rats, so the differences in EF% and FS% due to heart rates among those groups could be excluded. In comparison with those in the Sham group, the chambers of the heart were enlarged, and the cardiac wall was thinned in the I/R group, while the knockdown of lncRNA MALAT1 could prominently ameliorate the I/R-induced abnormal changes in the ventricular wall structure of the rats. Furthermore, FS% and EF% in each group were measured. The results revealed that the decreases in FS% and EF% in the I/R group were reversed remarkably after the knockdown of ln-

cRNA MALAT1 (*p*<0.05), manifesting that the knockdown of lncRNA MALAT1 can significantly improve the I/R-induced cardiac dysfunction.

Myocardial Infarction Area in Rats Reduced by Knockdown of LncRNA MALAT1

The myocardial infarction area was evaluated through the TTC staining. As shown in Figure 4, there were statistically significant differences in the myocardial infarction area among the three groups of rats [(1.23±0.52) *vs.* (56.93±1.45) *vs.* (21.19±1.47)] (*p*<0.05).

Myocardial I/R-Induced Pathological Injury Alleviated by Knockdown of LncRNA MALAT1

H&E staining was performed for the myocardial tissues to assess the microstructural changes of the myocardial cells in the cross-section of the heart in each group. According to Figure 5,

there was apparent edema in the myocardial cells, the myofilaments were disorderly arranged, and degradation and different degrees of necrosis occurred in the I/R group. After knockdown of lncRNA MALAT1, however, the cardiac tissue edema in the rats was relieved evidently, and the myofilament abnormality was improved markedly, suggesting that repressing lncRNA MALAT1 can prominently alleviate the I/R-induced myocardial injury in the rats.

Effects of LncRNA MALAT1 on Myocardial Cell Apoptosis in the Rats

The apoptosis level of the three groups of myocardial cells was determined using TUNEL staining. It was shown that the number of apoptotic myocardial cells in the rat myocardial tissues was increased notably after I/R injury (*p*<0.05), which was approximately (4.02±1.78) times that of the Sham group. However, the number of apoptotic myocardial cells in the rat myocardial
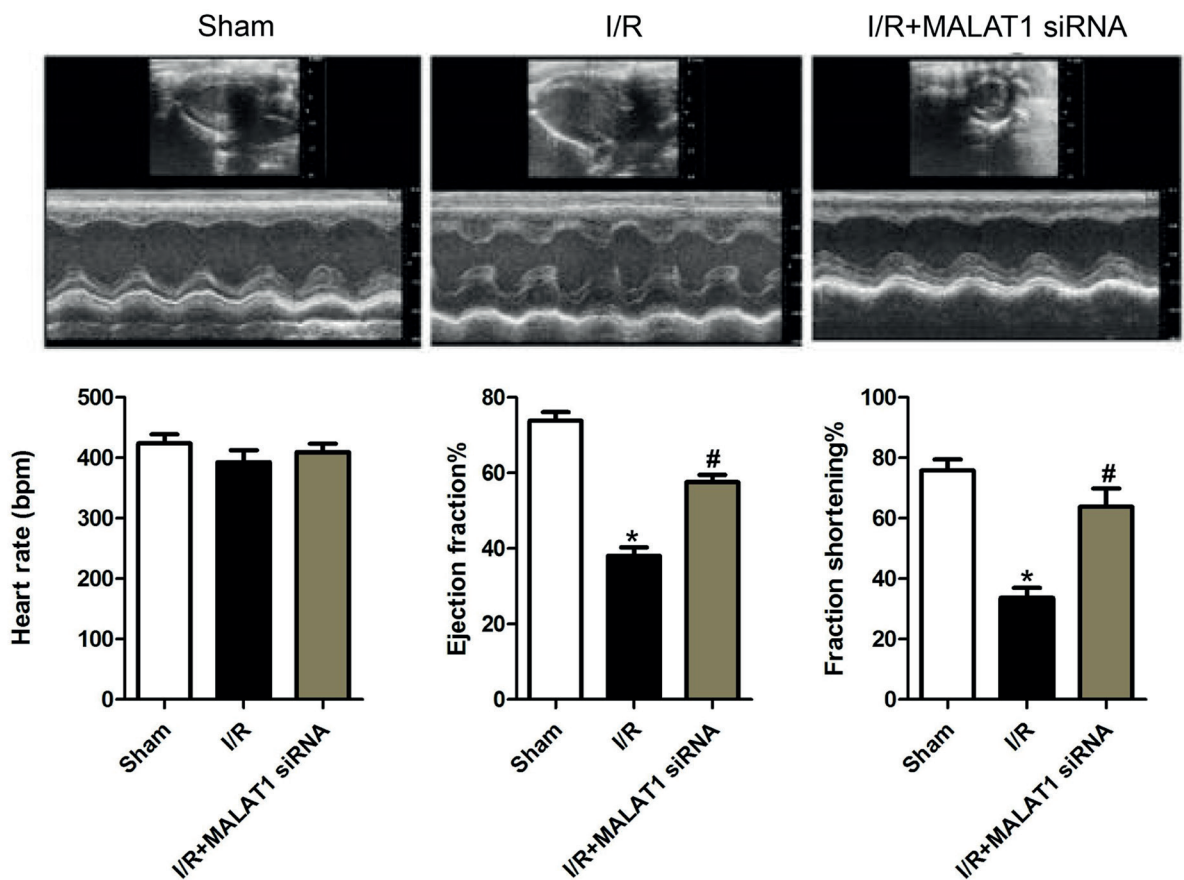
Figure 3. Effects of lncRNA MALAT1 on the cardiac function of the rats with myocardial I/R injury. Sham: Sham group, I/R: I/R group, I/R + MALAT1 siRNA: I/R + MALAT1 knockdown group, *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group, with statistical differences.
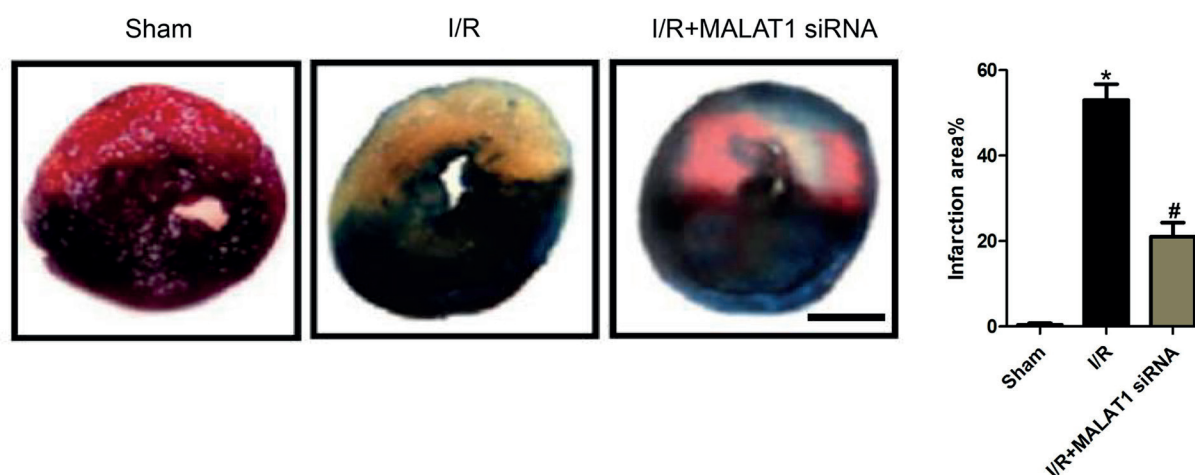
Figure 4. Myocardial infarction area in rats reduced by knockdown of lncRNA MALAT1. Sham: Sham group, I/R: I/R group, I/R + MALAT1 siRNA: I/R + MALAT1 knockdown group, *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group, with statistical differences.

tissues was decreased to (2.42±0.83) times that of the Sham group after inhibition on lncRNA MALAT1 (p<0.05), manifesting that the myocardial cell apoptosis can be significantly inhibited by repressing the expression of lncRNA MALAT1 (Figure 6).

Effects of LncRNA MALAT1 on Expressions of Apoptosis-Related Genes in the Myocardial Tissues of Rats

In addition, the expression levels of apoptosis-related proteins [B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax)] in the myocardial tissues of each group of rats were detected *via* Western blotting assay. The results (Figure 7) indicated that the ratio of Bax/Bcl-2 in the cardiac tissues in the I/R group was remarkably higher than that in the Sham group, but the ratio was decreased evidently after lncRNA MALAT1 in the cardiac tissues was knocked down, illustrating

that the apoptosis level of the myocardial cells is inhibited significantly (p<0.05).

### Expression of Ð-Catenin in the Myocardial Tissues in the Three Groups of Rats

Considering the important role of the β-catenin signaling pathway in the myocardial I/R injury, the expression level of β-catenin protein in the myocardial tissues in each group was measured. It was shown that the knockdown of lncRNA MALAT1 could significantly reverse the decline in the expression of the β-catenin protein in the cardiac tissues of the rats with I/R injury (p<0.05).

Discussion

Mechanical method or medical intervention is the most efficient strategy for rapidly restoring the blood flow passing through the occluded cor-
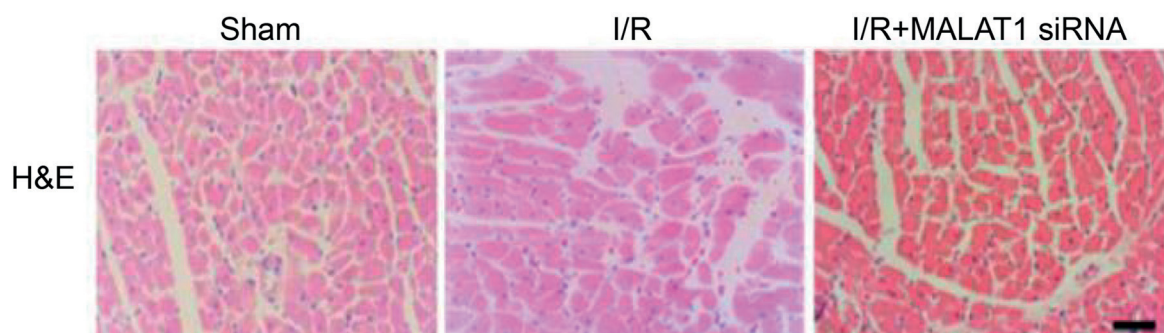
Figure 5. Myocardial I/R-induced pathological injury alleviated by knockdown of lncRNA MALAT1 (magnification: 100×). Sham: Sham group, I/R: I/R group, I/R + MALAT1 siRNA: I/R + MALAT1 knockdown group.
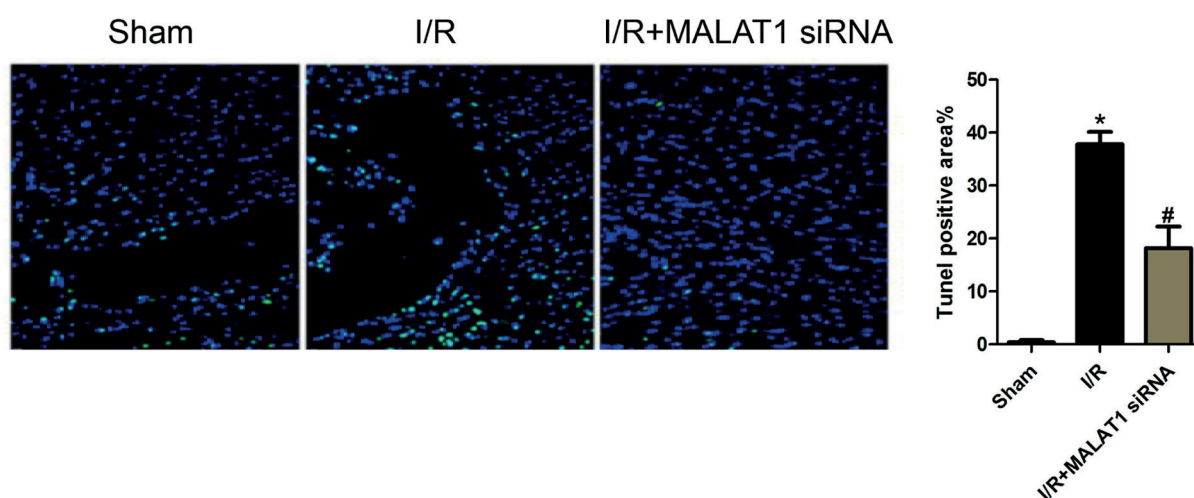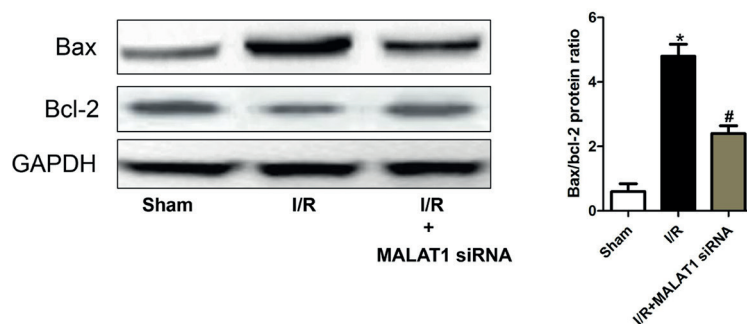
Figure 6. Effects of lncRNA MALAT1 on myocardial cell apoptosis in the rats (magnification: 40×). Sham: Sham group, I/R: I/R group, I/R + MALAT1 siRNA: I/R + MALAT1 knockdown group, *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group, with statistical differences.

onary artery, restricting the myocardial infarction area and improving the clinical prognosis of acute myocardial infarction[14]. Nevertheless, reperfusion itself will also cause additional myocardial cell death and increase the myocardial infarction area simultaneously. Factors leading to the reperfusion injury mainly include oxidative stress, inflammation and apoptosis. The ATP consumption in the myocardial cells during myocardial ischemia reduces the uptake of Ca^{2+} by the sarcoplasmic reticulum[15,16], thus triggering the massive accumulation of mitochondrial Ca^{2+}. During reperfusion, the oxygen enters into the myocardial cells again, causing damage to the mitochondrial electron transport chain and increasing the generation of reactive oxygen species (ROS)[17]. Both mitochondrial Ca^{2+} overload and increased ROS generation

can promote the opening of mitochondrial permeability transition pore and lead to cell energy metabolism disorder, ultimately triggering irreversible call necrosis and apoptosis[18,19]. Therefore, inhibiting the myocardial cell apoptosis, inflammation and oxidative stress during reperfusion can effectively ameliorate the I/R injury-induced abnormal cardiac function and reduce the myocardial infarction area at the same time.

The molecular mechanisms of lncRNA MALAT1, a member of the lncRNA family, in the occurrence and development of various diseases have been gradually revealed in recent years. For instance, lncRNA MALAT1 is capable of enhancing the oncogenic activity of EZH2 in castration-resistant prostate cancer[20]. Moreover, lncRNA MALAT1 can accelerate the occurrence and devel-

Figure 7. Effects of lncRNA MALAT1 on expressions of apoptosis-related genes in the myocardial tissues of rats. Sham: Sham group, I/R: I/R group, I/R + MALAT1 siRNA: I/R + MALAT1 knockdown group, *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group, with statistical differences.

Figure 8. Expression of β-catenin in the myocardial tissues in the three groups of rats. Sham: Sham group, I/R: I/R group, I/R + MALAT1 siRNA: I/R + MALAT1 knockdown group, *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group, with statistical differences.
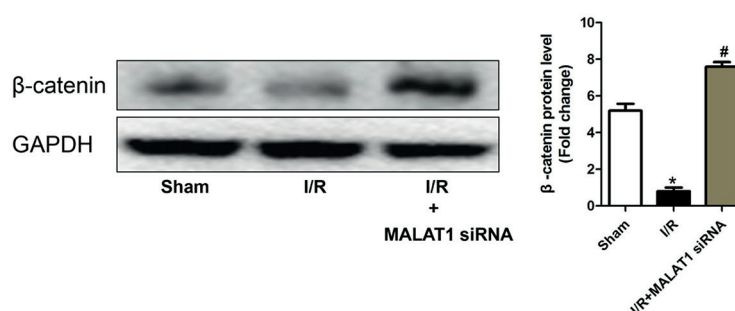
opment of lung adenocarcinoma by targeting the inhibition on miR-204[21]. Tang et al[22] have revealed that lncRNA MALAT1 can protect the endothelium from ox-LDL-induced dysfunction by up-regulating the expressions of CXCR2 and Akt, the target genes of miR-22-3p. In the case of cardiovascular diseases, the inhibition on lncRNA MALAT1 expression can decrease the myocardial cell apoptosis and improve the left ventricular function in rats with diabetic cardiomyopathy[23]. In sepsis-induced cardiomyopathy[24], lncRNA MALAT1 is also able to aggravate cardiac inflammation and dysfunction by influencing miR-125b and the p38 MAPK/NF-κB signaling pathway. Vausort et al[25] reported in the literature that MALAT1 is highly expressed in patients with acute myocardial infarction. Autophagy is a degradation process of intracellular proteins and metabolites, which plays a crucial role in maintaining cellular homeostasis. However, the hyperactivation of autophagy may induce autolysis and cell death, so autophagy exerts important effects in multiple heart diseases, including myocardial I/R injury. MiR-204 can protect the myocardial cells from I/R injury by inhibiting the death of autophagic cells, and lncRNAs can interact with miRNAs and regulate their expressions as the endogenous miRNA sponge. Li et al[21] have reported that lncRNA MALAT1 contains the binding sites of miR-204, so it can serve as the endogenous sponge repressing the actions of miR-204. According to the results in this research, the expression level of lncRNA MALAT1 in the myocardial tissues of the rats with I/R injury was elevated markedly, while the knockdown of lncRNA MALAT1 with siRNA could prominently ameliorate the I/R injury-induced cardiac dysfunction of the rats, decrease the myocardial infarction area, alleviate pathological damage to the myocardial tissues, reduce the number of apoptotic myocardial cells and repress the expression of apoptosis-related proteins. Furthermore, the results of Western blotting assay revealed that the impacts of lncRNA MALAT1 on the myocardial cell apoptosis and cardiac function

in the rats with I/R injury might be influenced by its regulatory effect on the β-catenin protein. As described above, it was speculated that the knockdown of lncRNA MALAT1 possibly attenuates its sponging effect on miR-204, thereby up-regulating the expression level of miR-204 and finally inhibiting the myocardial cell apoptosis. Nonetheless, there were still some limitations in this research: 1) No cell experiments were established for *in vitro* verification. 2) The direct action targets of lncRNA MALAT1 were not verified. 3) Inhibition tests were not designed to explore whether the effects of lncRNA MALAT1 on the myocardial I/R injury is dependent on β-catenin.

Conclusions

It is revealed in this research for the first time that the knockdown of lncRNA MALAT1 can improve the cardiac function and inhibit the apoptosis level of myocardial cells in the rats with I/R injury by up-regulating the expression of the β-catenin protein. Therefore, lncRNA MALAT1 is expected to become an important target for the treatment of myocardial infarction.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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