

Original Article

MiR-22 inhibits myocardial fibrosis in rats with myocardial infarction by targeting PTEN/Akt/mTOR signaling pathway

Bolin Li¹, Chunna Liang¹, Yuanjun Lv¹, Yongjin Tan², Weiyan Chen^{3,*}¹ Department of Emergency, Kaiping Central Hospital, Jiangmen, China² Department of Cardiovascular, Kaiping Central Hospital, Jiangmen, China³ Intensive Care Unit, the Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, China

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Abstract

This study aimed to evaluate the effects of miR-22 on myocardial fibrosis in rats with myocardial infarction (MI) and to further explore the possible underlying mechanism. A total of 80 rats were randomly divided into Sham group, miR-22 overexpression group, MI group or MI + miR-22 overexpression group. Reverse transcription-polymerase chain reaction (RT-PCR) results showed that compared with Sham group, miR-22 expression level in myocardial tissues of rats decreased significantly in MI group. Overexpression of miR-22 could remarkably relieve cardiac insufficiency in MI rats, increase EF% and FS%, and reduce collagen deposition and the mRNA expression level of fibrosis-promoting genes in myocardial tissues of MI rats. The cross-sectional area of myocardial cells in MI + miR-22 mimic group was smaller than that in MI group. According to the results of immunohistochemical staining, overexpression of miR-22 notably reduced the level of oxidative stress marker 4-HNE in myocardial tissues of MI rats. Meanwhile, myocardial cells in MI + miR-22 mimic group exhibited a prominently lower apoptosis rate than those in MI group. Furthermore, Western blotting results demonstrated that overexpression of miR-22 inhibited the activation of the phosphatase and tensin homolog deleted on chromosome ten (PTEN)/Akt/mTOR signaling pathway in myocardial tissues of MI rats. The inhibitory effects of miR-22 on myocardial fibrosis and hypertrophy after MI in rats may be related to its inhibition on the PTEN/Akt/mTOR signaling pathway. All our findings suggested that miR-22 is expected to become a targeted drug for the clinical treatment of MI.

Keywords: Myocardial infarction, Fibrosis, MiR-22, PTEN

1. Introduction

With the aging of the population, heart failure has become an increasingly serious public health issue worldwide. Heart failure is a common pathological manifestation of diverse cardiovascular diseases, such as ischemic cardiomyopathy, myocardial infarction (MI), valvular heart disease, hypertension, myocarditis, cardiomyopathy, diabetes and other end-stage diseases [1-3]. A series of pathological changes after MI, including hypertrophy of myocardial cells, extracellular matrix remodeling, insufficient microvascular perfusion and ventricular dilatation, act as crucial factors leading to the occurrence and development of heart failure [4,5]. Hence, it is of great significance to clarify the molecular mechanisms of the occurrence and development of MI for its clinical prevention and treatment.

Micro ribonucleic acids (miRNAs) are a group of single-stranded non-coding RNAs with about 20-24 nucleotides in length. They exist in eukaryotes and possess regulatory functions. Previous studies have shown that miRNAs can regulate the expression of different life genes through targeted binding to specific genes, thereby playing important roles in cell proliferation, differentiation, inva-

sion, apoptosis, and other behaviors [6,7]. Moreover, miRNAs exert vital effects in cardiovascular diseases, especially heart failure and MI. For example, miR-1 triggers arrhythmia by modulating potassium channel genes. Up-regulated miR-1 in the serum of MI patients is a high-risk factor for arrhythmia [8]. Transgenic mice with myocardial cells overexpressing miR-199b-5p display obvious pathological myocardial remodeling after MI, accompanied by severe cardiac contraction, diastolic dysfunction and fibrotic deposition [9]. Although miR-22 is the first miRNA discovered in HeLa cells in Lagos, there is still no research on its functions and mechanisms in MI.

In this study, the expression of miR-22 in myocardial tissues of MI rats was first detected. Meanwhile, the potential mechanism by which miR-22 exhibited its anti-MI effect was explored. All our findings might help to provide a certain reference basis for the clinical treatment of MI in the future.

2. Materials and Methods

2.1. Grouping and treatments of experimental animals

A total of 80 adult male Wistar rats at the age of 12-

* Corresponding author.

E-mail address: sam11124@126.com (Weiyan Chen).Doi: <http://dx.doi.org/10.14715/cmb/2024.70.1.4>

14 weeks old weighing (267.53±8.29) g were randomly divided into 4 groups using a random number table, namely, sham operation group (Sham group), sham operation + miR-22 overexpression group (Sham + miR-22 mimic group), MI group, MI + miR-22 overexpression group (MI + miR-22 mimic group). There were no statistically significant differences in basic data such as age and weight among the 4 groups of rats. Rats in each group were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital. Then the cannula was inserted into the left carotid artery to measure the blood pressure of rats. Limb lead II electrocardiogram (ECG) was used to detect heart rate. Subsequently, the thorax in the fourth intercostal space was opened, and the pericardium was cut off to expose the heart. Next, the left anterior descending coronary artery (LAD) was ligated at 2 mm above the left atrial appendage using 6-0 silk thread to induce local MI model. If ST segment was elevated in limb lead II ECG, and the myocardium in the heart's left ventricle anterior wall turned white, MI modeling would be successful. In addition, the rats in Sham group underwent the same operation, without ligation of the silk thread. At 4 weeks after operation, the rats were killed, and myocardial tissues in the left ventricle anterior wall were collected. After washing with normal saline to remove blood, collected tissues were put into a refrigerator at -80°C for use (Figure 1). Rats in Sham + miR-22 mimic group and MI + miR-22 mimic group were injected with miR-22 mimic *via* the tail vein once every 5 days for 4 weeks after operation. Meanwhile, those in Sham group and MI group were injected with the same amount of normal saline. This investigation was approved by the Animal Ethics Committee of the Second Affiliated Hospital of Guangzhou Medical University Animal Center.

2.2. Detect via ECGs

To detect the cardiac function, Mylab 30CV ultrasound system (Esaote S.p.A, Genoa, Italy) and 10-MHz linear ultrasound transmitter were applied to detect the ECGs of each group of rats. Briefly, the hair in the anterior thoracic region was first shaved off. Then the rats were anesthetized and placed on a heating plate (37°C) with the left side facing up. Parameters, including ejection fraction [EF (%)], fraction shortening [FS (%)] and heart rate (bpm), were finally detected.

2.3. Hematoxylin and Eosin (H&E) staining

The hearts of each group were placed in 10% formalin overnight, dehydrated and embedded with paraffin blocks. Subsequently, myocardial tissues were cut into 5 µm-thick slices, fixed on glass slides, and baked dry for staining. According to the instructions, the slices were independently soaked in xylene, ethanol with a gradient concentration and hematoxylin, followed by sealing with resin. After air drying, the slices were observed and photographed under an optical microscope. Finally, the morphology of myocardial cells, cardiac interstitium and myofilaments was observed.

2.4. Immunohistochemical staining

Myocardial tissue slices were baked in an oven at 60°C for 30 min and deparaffinized with xylene (5 min × 3 times), followed by dehydration for 3 times with 100%, 95% and 70% ethanol, respectively. Thereafter, the activity

of endogenous peroxidase was inhibited by 3% hydrogen peroxide methanol, and the tissues were sealed with goat serum for 1 h. Then the tissues were incubated with the primary antibody against 4-hydroxynonenal (4-HNE) diluted with phosphate-buffered saline (PBS) at 1:100 at 4°C overnight. After washing with PBS for 4 times in a shaker, the tissues were incubated with the corresponding secondary antibody. Color development was performed with diaminobenzidine. 6 samples were randomly selected from each group. 5 fields of view were randomly selected from each sample and photographed under an optical microscope (200× and 400×).

2.5. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) staining

Myocardial tissue slices were baked in an oven at 60°C for 30 min, deparaffinized with xylene (5 min × 3 times) and independently dehydrated with 100%, 95% and 70% ethanol for 3 times each. Subsequently, the slices were incubated with protein kinase K for half an hour. After washing with PBS, the slices reacted with dUTP labeled by terminal deoxynucleotidyl transferase and luciferase for 1 h at 37°C. Next, the specific antibody labeled by horseradish peroxidase was added for incubation again for 1 h at 37°C. The slices reacted at room temperature for 10 min, with diaminobenzidine (DAB) (Solarbio, Beijing, China) as the substrate. The nucleus was stained by hematoxylin, and photographing and counting were carried out under an optical microscope.

2.6. Picro-Sirius Red (PSR) staining

Myocardial tissue slices were baked in an oven at 60°C for 30 min, deparaffinized with xylene (5 min × 3 times), and separately dehydrated with 100% ethanol, 95% ethanol and 70% ethanol for 3 times each. Next, myocardial tissues were added drop-wise with Sirius red stain for 1 h of incubation, followed by washing with running water for 10 min to remove staining solution. After the nucleus was stained by hematoxylin, the tissues were mounted and observed.

2.7. Detection of related gene expression via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs in myocardial tissues of 4 groups of rats were first extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNAs were detected by an ultraviolet spectrophotometer. When $A_{260}/A_{280} = 1.8-2.0$, the RNA could be used. Subsequently, extracted RNA was reversely transcribed into complementary deoxyribonucleic acids (cDNAs) and stored in a refrigerator at -80°C. RT-PCR was then carried out in a system containing 2.5 µL of 10× Buffer, 1 µL of cDNA, 0.5 µL of forward primers (20 µmol/L), 0.5 µL of reverse primers (20 µmol/L), 10 µL of LightCycler® 480 SYBR Green I Master (2×) and 5.5 µL of ddH₂O. RT-PCR amplification systems were the same as above. U6 was used as the internal reference in the quantitative analysis of miR-22 expression, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference in the quantitative analysis of the Collagen1 and Collagen3 expression. Primer sequences used in the study are shown in Table 1.

Table 1. Primer sequences of each index in RT-PCR.

Target gene		Primer sequence
GAPDH	Forward	5'-GACATGCCCGCCTGGAGAAAC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGT-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'
MiR-22	Forward	5'-AGCAGTCGATGCTGGTGA-3'
	Reverse	5'-AGCTACGCTAGTTTTTCACCC-3'
Collagen1	Forward	5'-TGCTAAAGCTGTCCCGATGCTG-3'
	Reverse	5'-CGATCGTAGTCGTAGGATGCTC-3'
Collagen3	Forward	5'-CGATGCTGTATAGTCGTAGTGC-3'
	Reverse	5'-ACGTAGTCGTTAGTCGTAGTCA-3'

2.8. Detection of protein expression via Western Blotting assay

After myocardial tissues were fully ground in lysis buffer, they were ultrasonically lysed. The lysis buffer was centrifuged to extract the supernatant, which was then sub-packaged into Eppendorf (EP) tubes for use. Protein concentration was measured *via* the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and ultraviolet spectrophotometry. After that, proteins were dissolved to a constant concentration and placed in a refrigerator at -80°C. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto cellulose acetate polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary antibodies at 4°C overnight, the membranes were incubated with goat anti-rabbit secondary antibody for 1 h in dark. Immuno-reactive bands were scanned using an Odyssey scanner and quantified. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

2.9. Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean \pm standard deviation. *t*-test was applied to compare the differences between the two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. MiR-22 expression in myocardial tissues of the 4 groups of rats

RT-PCR results (Figure 1) revealed that the expression level of miR-22 in myocardial tissues of rats in MI group was significantly lower than (about 0.33 times as high as) that in Sham group ($P < 0.05$). Compared with MI group, the expression level of miR-22 in myocardial tissues of rats in MI + miR-22 mimic group increased obviously ($P < 0.05$). This indicated that the model of miR-22 overexpression was successfully established in rats.

3.2. Comparison of cardiac function among the 4 groups of rats

As shown in Figure 2, ECGs manifested that there was no statistically significant difference in heart rate among the 4 groups of rats. Therefore, it could be concluded that differences in EF (%) and FS (%) in each group of rats were not caused by the difference in heart rate. In comparison with Sham group, the heart of rats in MI group was enlarged and the heart wall became thinner, which could

be markedly improved by miR-22 overexpression. Subsequently, FS (%) and EF (%) in each group of rats were measured. The results manifested that overexpression of miR-22 evidently inhibited the decreases in FS (%) and EF (%) in MI group ($P < 0.05$). All these findings suggested that miR-22 could improve deteriorated cardiac functions of MI rats.

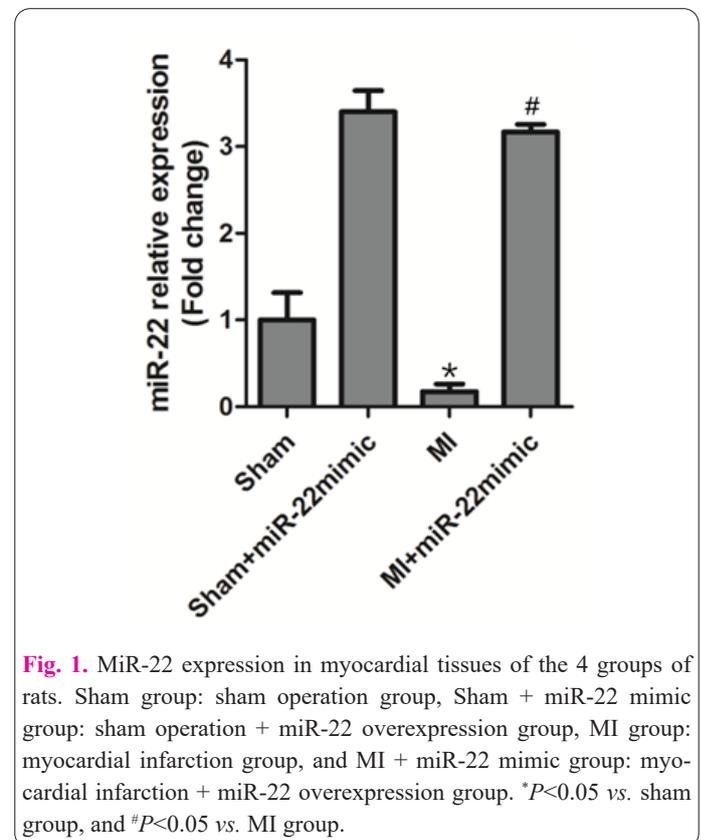


Fig. 1. MiR-22 expression in myocardial tissues of the 4 groups of rats. Sham group: sham operation group, Sham + miR-22 mimic group: sham operation + miR-22 overexpression group, MI group: myocardial infarction group, and MI + miR-22 mimic group: myocardial infarction + miR-22 overexpression group. * $P < 0.05$ vs. sham group, and # $P < 0.05$ vs. MI group.

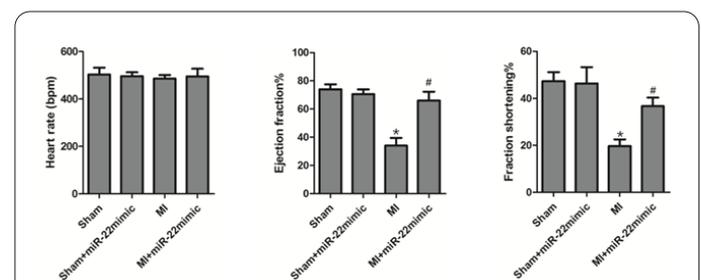


Fig. 2. Comparison of cardiac function among the 4 groups of rats. Sham group: sham operation group, Sham + miR-22 mimic group: sham operation + miR-22 overexpression group, MI group: myocardial infarction group, and MI + miR-22 mimic group: myocardial infarction + miR-22 overexpression group. * $P < 0.05$ vs. sham group, and # $P < 0.05$ vs. MI group.

3.3. Comparison of myocardial fibrosis among the 4 groups of rats

PSR staining results (Figure 3) demonstrated that the content of deposited collagens in myocardial tissues of rats in MI + miR-22 mimic group was prominently lower than that in MI group ($P < 0.05$). RT-PCR results further revealed that overexpression of miR-22 markedly suppressed the mRNA expressions of fibrosis-promoting genes (Collagen1 and Collagen3) ($P < 0.05$).

3.4. Comparison of myocardial hypertrophy among the 4 groups of rats

H&E staining results (Figure 4) indicated that overexpression of miR-22 remarkably reduced the cross-sectional area of myocardial cells after MI ($P < 0.05$), suggesting that miR-22 had an anti-myocardial hypertrophy effect.

3.5. Comparison of oxidative stress level in myocardial tissues among the 4 groups of rats

Furthermore, the protein expression level of lipid peroxide 4-HNE was determined in myocardial tissues of the 4 groups of rats. The results showed that rats in MI + miR-22

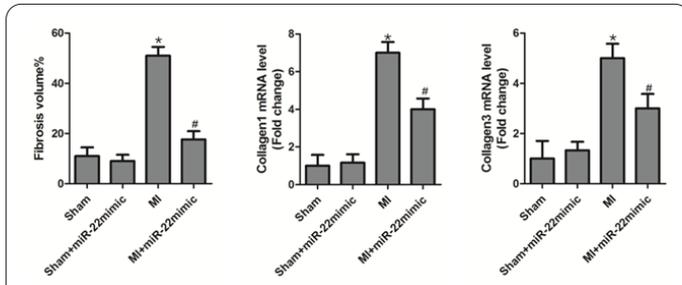


Fig. 3. Comparison of myocardial fibrosis among the 4 groups of rats, (magnification: 400×). Sham group: sham operation group, Sham + miR-22 mimic group: sham operation + miR-22 overexpression group, MI group: myocardial infarction group, and MI + miR-22 mimic group: myocardial infarction + miR-22 overexpression group. * $P < 0.05$ vs. sham group, and # $P < 0.05$ vs. MI group.

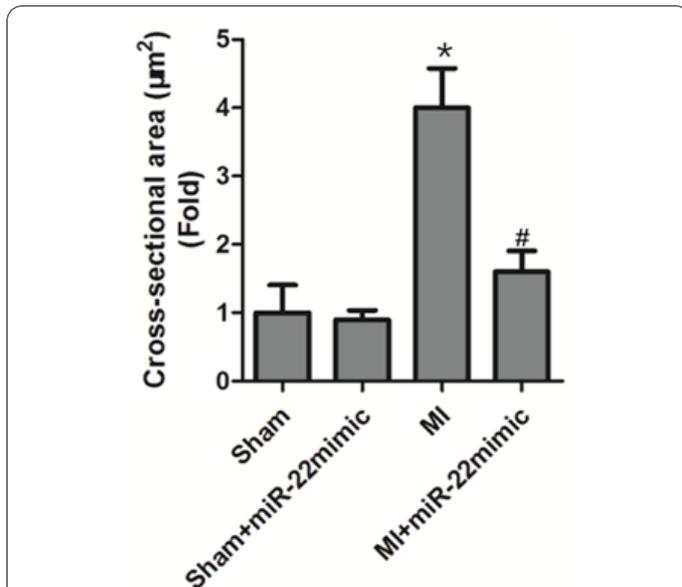


Fig. 4. Comparison of myocardial hypertrophy among the 4 groups of rats, (magnification: 400×). Sham group: sham operation group, Sham + miR-22 mimic group: sham operation + miR-22 overexpression group, MI group: myocardial infarction group, and MI + miR-22 mimic group: myocardial infarction + miR-22 overexpression group. * $P < 0.05$ vs. sham group, and # $P < 0.05$ vs. MI group.

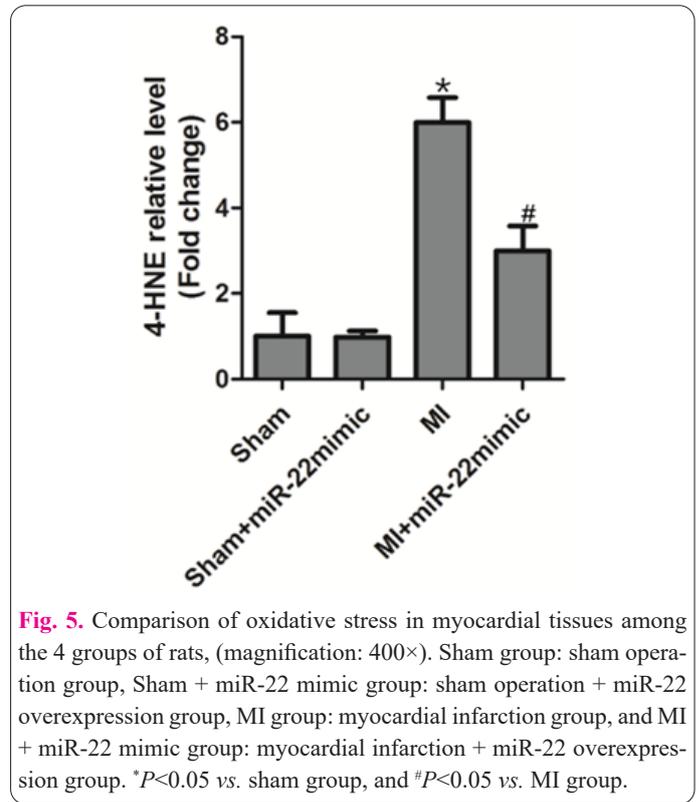


Fig. 5. Comparison of oxidative stress in myocardial tissues among the 4 groups of rats, (magnification: 400×). Sham group: sham operation group, Sham + miR-22 mimic group: sham operation + miR-22 overexpression group, MI group: myocardial infarction group, and MI + miR-22 mimic group: myocardial infarction + miR-22 overexpression group. * $P < 0.05$ vs. sham group, and # $P < 0.05$ vs. MI group.

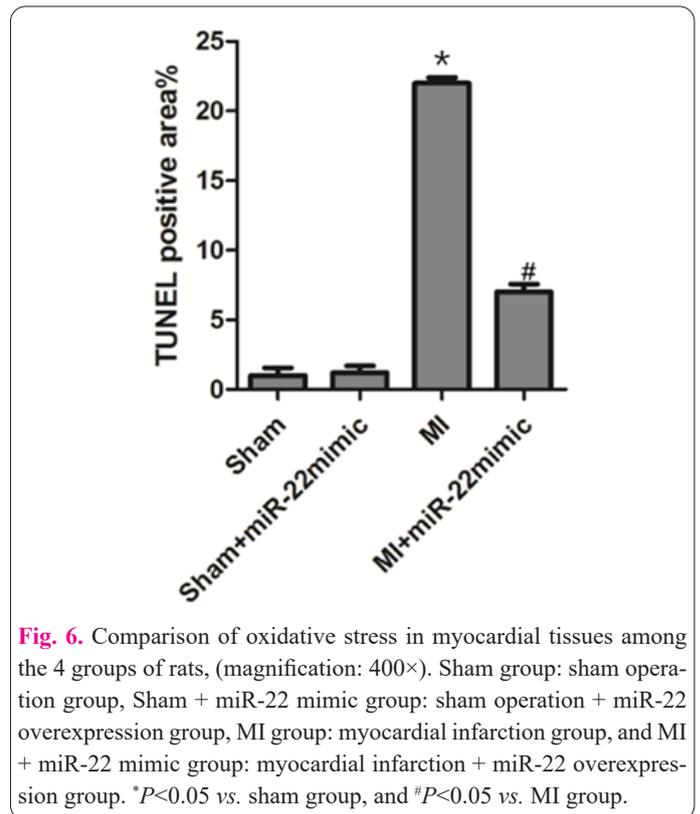


Fig. 6. Comparison of oxidative stress in myocardial tissues among the 4 groups of rats, (magnification: 400×). Sham group: sham operation group, Sham + miR-22 mimic group: sham operation + miR-22 overexpression group, MI group: myocardial infarction group, and MI + miR-22 mimic group: myocardial infarction + miR-22 overexpression group. * $P < 0.05$ vs. sham group, and # $P < 0.05$ vs. MI group.

22 mimic group exhibited a significantly lower content of 4-HNE in myocardial tissues than MI group ($P < 0.05$) (Figure 5).

3.6. Comparison of the apoptosis rate in myocardial tissues among the 4 groups of rats

TUNEL staining results (Figure 6) illustrated that the apoptosis rate in myocardial tissues of rats increased significantly after MI ($P < 0.05$). However, miR-22 overexpression distinctly decreased the number of apoptotic myocardial cells of MI rats ($P < 0.05$).

3.7. Expression level of genes associated with the PTEN/Akt/mTOR signaling pathway

Considering that the abnormal activation of the PTEN/Akt/mTOR signaling pathway is a key molecular mechanism triggering MI, Western blotting assay was adopted to detect the expression of proteins related to the PTEN/Akt/mTOR signaling pathway. As shown in Figure 7, overexpression of miR-22 obviously inhibited the phosphorylation levels of Akt and mTOR in myocardial tissues, and significantly reduced the protein expression of PTEN as well ($P < 0.05$).

4. Discussion

After MI, ventricles will undergo progressive enlargement and shape change, which is called ventricular structural remodeling. Similarly, abnormal changes in electrical activity and metabolic activity of myocardial cells after MI are known as electrical remodeling and metabolic remodeling [10]. Post-MI structural remodeling, electrical remodeling and metabolic remodeling are all vital incentives for heart failure in MI patients. The emphasis of current research is mainly on post-MI structural remodeling. It has been recognized that ventricular structural remodeling primarily involves changes in heart cells (including myocardial cells, fibroblasts and immune cells) and abnormalities in the extracellular matrix [11]. Among them, the dynamic generation and degradation of collagen fibers in myocardial interstitium are valuable for the maintenance of normal cardiac activities. After MI, the destruction of dynamic balance induces myocardial fibrosis. This may reduce ventricular compliance and myocardial contractility, thereby leading to heart failure [12]. Additionally, collagen deposition in myocardial fibrosis damages myocardial cell electrical homeostasis and triggers arrhythmia [13]. Hence, it is of great significance to further explore MI, especially the core molecular targets of myocardial fibrosis after MI, for the research and development of targeted drugs for its prevention and treatment.

MiRNAs are a type of small non-coding RNAs with about 20-24 nucleotides in length, which can modulate the expression of various genes in the body at the post-transcriptional level. More precisely, miRNAs are able to suppress the translation of corresponding proteins by binding to the 3' untranslated region (3'UTR) of target genes [14]. MiR-22, belonging to the miRNA family, plays a crucial regulatory role in cardiovascular diseases, especially myocardial remodeling. Previous studies have shown that miR-22 is a pivotal agonist of calcium ion (Ca^{2+})-ATPases in myocardial cells, whose activity in the sarcoplasmic reticulum/endoplasmic reticulum can be reduced by inhibition of miR-22 expression. This may cause the reduction in the content of Ca^{2+} in the sarcoplasmic reticulum of myocardial cells, eventually resulting in systolic and diastolic dysfunction of the heart as well as cardiac cavity enlargement [15]. Patients with end-stage heart failure display a markedly up-regulated expression level of miR-22 in the peripheral blood. Animal experiments have further illustrated that miR-22 can target PPAR and nuclear estrogen receptor genes, which destructs calcium homeostasis in myocardial cells and even induces heart failure [16]. In this study, it was discovered that the expression level of miR-22 was significantly reduced in myocardial tissues of MI rats. Furthermore, miR-22 overexpression could inhibit myocardial hypertrophy and fibrosis after MI and

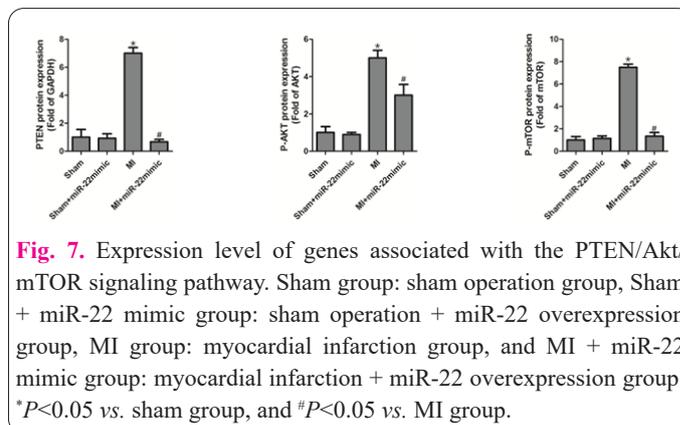


Fig. 7. Expression level of genes associated with the PTEN/Akt/mTOR signaling pathway. Sham group: sham operation group, Sham + miR-22 mimic group: sham operation + miR-22 overexpression group, MI group: myocardial infarction group, and MI + miR-22 mimic group: myocardial infarction + miR-22 overexpression group. * $P < 0.05$ vs. sham group, and # $P < 0.05$ vs. MI group.

reduce myocardial cell apoptosis.

There are numerous and complex signaling pathways giving rise to myocardial remodeling after MI. Physiological myocardial hypertrophy is mainly mediated by PI3K, whose downstream target protein is Akt. Besides, phosphorylated Akt is able to activate mTOR that is the core of physiological myocardial hypertrophy [17]. On the contrary, pathological myocardial hypertrophy (stimulated by angiotensin II or endothelin-1) is mediated by the activation of G protein-coupled receptor. Activated G protein-coupled receptor can lead to the activation of MAPK and the dephosphorylation of NFAT, as well as facilitate the transcriptional activation of hypertrophy-associated genes [18]. Relevant studies have demonstrated that phosphatase PTEN gives rise to remodeling after MI through the Akt/IL-10 signaling pathway. Therefore, suppressing PTEN in a targeted manner can alleviate post-MI myocardial remodeling [19]. However, miR-22 can target and inhibit PTEN gene at a specific site on PTEN 3'UTR. More interestingly, miR-22 can be up-regulated by PTEN, implying that there is a potential negative feedback regulation loop between miR-22 and PTEN/Akt [20-23]. The results of this study revealed that the expression levels of PTEN, Akt and mTOR were significantly up-regulated in myocardial tissues of MI rats. In addition, overexpression of miR-22 could prominently reduce the expression and activation of PTEN, Akt and mTOR in myocardial tissues. Nevertheless, whether there is a potential negative feedback regulation correlation between miR-22 and PTEN/Akt needs to be confirmed by more experiments in the future.

5. Conclusions

To sum up, our results demonstrated for the first time that the miR-22 expression was reduced in myocardial tissues of MI rats. Furthermore, miR-22 could reduce myocardial fibrosis after MI through the targeted inhibition on the PTEN-mediated Akt/mTOR signaling pathway.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This investigation was approved by the Animal Ethics Committee of the Second Affiliated Hospital of Guang-

zhou Medical University Animal Center.

Informed consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions

Bolin Li and Weiyang Chen designed the study and performed the experiments, Chunna Liang and Yuanjun Lv collected the data, Chunna Liang, Yuanjun Lv and Yongjin Tan analyzed the data, Bolin Li and Weiyang Chen prepared the manuscript. All authors read and approved the final manuscript.

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