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MicroRNA-221 promotes myocardial apoptosis caused by myocardial ischemia-reperfusion by down-regulating PTEN

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether microRNA-221 could promote cardiomyocyte apoptosis by down-regulating the expression of PTEN (gene of phosphate and tension homology deleted on chromosome ten), thereby participating in the development of myocardial ischemia-reperfusion.

MATERIALS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to analyze the expression levels of microRNA-221 and PTEN in human cardiomyocytes (HCM) cells treated with hypoxia/reoxygenation (H/R). The expressions of myocardial injury markers, including lactic dehydrogenase enzyme (LDH), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) were determined by qRT-PCR as well. The binding relationship between microRNA-221 and PTEN was verified by the Dual-Luciferase reporter gene assay. Subsequently, microRNA-221 inhibitor and si-PTEN were transfected into cells. The proliferation and apoptosis of cells were analyzed using Cell Counting Kit-8 (CCK-8) and flow cytometry, respectively. In addition, the expression levels of apoptosis-related proteins were determined by Western blot.

RESULTS: The qRT-PCR results confirmed that the expression level of microRNA-221 in H/R treated cells was significantly up-regulated when compared with the normoxic treated group, whereas PTEN expression was markedly down-regulated. After silencing microRNA-221, the expression levels of myocardial injury markers, including LDH, MDA, GSH-PX in H/R cells were significantly decreased. However, SOD levels were remarkably increased. At the same time, down-regulation of microRNA-221 markedly increased cell proliferation, whereas decreased apoptosis. However, microRNA-221 enhanced the expression of apoptosis-related genes, including Bax and cytochrome C. Meanwhile, the expression level of anti-apoptotic gene Bcl-2 was significantly inhibited. The Dual-Luciferase reporter gene assay showed that microRNA-221 could target bind to PTEN and inhibit its expression. Similarly, down-regulation of PTEN markedly decreased cell proliferation and increased cell apoptosis. Furthermore, PTEN down-regulation remarkably promoted protein expression of pro-apoptosis-related genes, whereas inhibited the protein expression of anti-apoptotic genes.

CONCLUSIONS: MicroRNA-221 promoted myocardial apoptosis induced by myocardial ischemia-reperfusion by down-regulating PTEN. Therefore, microRNA-221 might be a potential therapeutic target for myocardial ischemia-reperfusion injury.

Key Words:
MicroRNA-21, PTEN, Ischemia-reperfusion, Myocardial apoptosis.

Introduction

Acute myocardial infarction (AMI) is an important cause of death in patients with cardiovascular diseases. The key to saving sudden cardiac muscle is to clear the infarct artery and to restore the blood supply of the heart. It has been found that myocardial ischemia after a certain period of time can aggravate the myocardial injury. This may eventually induce myocardial stunning, cardiac insufficiency, arrhythmia, etc., which is called myocardial ischemia-reperfusion injury[1,2]. Myocardial ischemia-reperfusion leads to the production of excessive oxygen free radicals and inflammatory factors in tissues. Eventually, this can cause apoptosis or necrosis of cells and tissue damage, and further induce ventricular remodeling and heart failure[3-5]. How to avoid myocardial ischemia-reperfusion injury after timely recovery of coronary blood flow has become a difficult problem in the treatment of cardiac ischemia. However, there is still no effective treatment for the ischemia-reperfusion injury. Therefore, it is

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of great significance to further explore new drugs and treatment methods.

MicroRNAs (miRNAs) are a class of endogenous non-coding small RNAs with about 21 to 23 bases in length. They can regulate gene expression by binding to the 3'untranslated regions (3'UTR) of downstream target genes, promoting mRNA degradation or inhibit mRNA translation. Eventually, they participate in cell growth, apoptosis, proliferation, migration and other biological behaviors[6]. Meanwhile, miRNAs are closely related to the development of various diseases. Studies[7,8] have confirmed that miRNAs are abundantly expressed in the cardiovascular system. It has been found that multiple miRNAs play an important role in the myocardial ischemia-reperfusion injury. For example, miR-34a regulates cardiomyocyte fibrosis, hypertrophy and apoptosis. However, miR-21 and miR-378 are closely related to ventricular remodeling and heart failure processes[9]. In addition, miR-19b is highly expressed in H2O2-induced H9c2 cardiomyocytes. Furthermore, down-regulation of miR-19b expression can significantly inhibit H2O2-induced apoptosis of H9c2 cardiomyocytes[10].

MicroRNA-221 (miRNA-221) has long been recognized as a cancer-associated gene. Previous studies have demonstrated that it is highly expressed in various tumors, such as lung cancer, breast cancer and gastric cancer. Besides, it is the same onco-miR as miR-222, both located on the X chromosome p11.3[11]. Moreover, authors[12] have also shown that microRNA-221 is highly expressed in plasma of patients with heart failure. It has also been confirmed that the expression of multiple circulating miRNAs in myocardial infarction is abnormal. Among them, the expression of circulating microRNA-221 is the highest, which is 3.89 times higher than that of the normal group. Furthermore, microRNA-221 is closely related to Synthax scores and myocardial injury markers, such as hypersensitive cardiac troponin T and GRACE[13]. These findings suggest that circulating microRNA-221 can be used as a new marker for early diagnosis of myocardial infarction. In addition, microRNA-221 is closely associated with diabetic myocardial oxidative stress[14]. It also exerts protective effects on H9c2 cell apoptosis induced by hypoxia and reoxygenation[15]. Therefore, we speculated, in this work, that microRNA-221 was closely related to myocardial oxidative stress injury. However, no research has investigated the mechanism of myocardial ischemia-reperfusion myocardial apoptosis. The purpose of this study was to investigate whether microRNA-221 was involved in myocardial apoptosis after myocardial ischemia-reperfusion injury.

Materials and Methods

Cell Culture and H/R Treatment

Human cardiomyocytes (HCM) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (Gibco, Grand Island, NY, USA). Hypoxia/reoxygenation (H/R) treatment of HCM cells was performed as follows. First, HCM cells in serum-free DMEM were placed in a humidified chamber equilibrated with 5% CO_2 and 95% N_2 for 4 hours, followed by re-oxygenation in 5% CO_2 and 95% of air for 3 hours in DMEM containing 10% glycerol. 24 hours after re-oxygenation, the gene expression and cell apoptosis condition were measured. Meanwhile, cells cultured under normal oxygen conditions were used as controls.

Cell Transfection

MicroRNA-221 inhibitor and PTEN (gene of phosphate and tension homology deleted on chromosome ten) siRNA, as well as the corresponding negative controls, were purchased from GenePharma (Shanghai, China). All cells were regularly cultured, and the medium was changed. When cell density was up to 60-70%, transfection reagent was gently mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), followed by incubation for 15 min at room temperature. Subsequently, the mixture and 300 μL of serum-free DMEM were added in cells. Fresh medium was added to replace the primary medium 4-6 h later. Then, the cells were cultured in an incubator with CO_2 at 37°C for 24 hours. Finally, transfection efficiency was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Extraction

Tissues or cells were lysed with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA), which were let stand for 5 min. 300 μL of chloroform was added to each Eppendorf tube (EP; Eppendorf, Hamburg, Germany) and mixed at room temperature. After incubation for 5 min, the tubes were centrifuged at 12000 r/s for 15 min at 4°C. Then, the supernatant was carefully pipetted into another new RNase free centrifuge tube. Afterward, isopropanol was added to the tubes at a ratio of 1:1. The mixture was then

gently mixed thoroughly and let stand at room temperature for 10 min. After centrifugation at 12000 r/s for 10 min at 4°C, RNA precipitate was collected and the supernatant was removed. 1 mL of 75% ethanol was added to the EP tubes, followed by centrifugation at 12000 g for 5 min at 4°C. Lastly, the precipitate was dissolved in 40 μL of Diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). The concentration of RNA was measured using a spectrophotometer, and extracted RNA was stored in a refrigerator at -80°C until use.

RNA Reverse Transcription and qRT-PCR Detection

Extracted total RNA was subjected to reverse transcription system in strict accordance with PrimeScript RT reagent Kit (TaKaRa, Code No. RR037A, Otsu, Shiga, Japan), and complementary deoxyribose nucleic acid (cDNA) was obtained. The reverse transcript template was added to RNase-depleted water to a final concentration of 10 ng/μL. Quantitative PCR operation was carried out in accordance with the instructions of SYBR Green PCR Kit (TaKaRa, Otsu, Shiga, Japan). The total reaction system was 10 μL. Primer sequences used in this study were as follows: microRNA-221 (F: 5'-GTTGGTGGGAGCTACATTGTCTGC-3'; R: 5'-GTGTCGTGGACTCGGCAATTC-3'); PTEN (F: 5'-TGGTGAGGTTTGATCCGCATA-3'; R: 5'-CCCAGTCAGAGGCGCTATG-3 '); Bax (F: 5'-CACAACTCAGCGCAAACATT-3'R; 5'ACAGCCATCTCTCTCCATGC-3'); Bcl2 (F: 5'GAAGCACAGATGGTTGATGG-3'; R: 5'-CAGCCTCACAAGGTTCCAAT-3'); Cytochrome C (F: 5'-TAAATATGAGGGTGTCGC-3'; R: 5'-AAGAATAGTTCCGTCCTG-3'); GAPDH (F: 5'-GGAATCCACTGGCGTCTTCA-3'; R: 5'-GGTTCACGCCCATCACAAAC-3'); U6 (F: 5'-AGAGAAGATTAGCATGGCCCCTG-3'; R: 5'-ATCCAGTGCGGGTCCGAGG-3').

Dual-Luciferase Reporting Assay

Bioinformatics was used to predict potential targets for microRNA-221 and PTEN. PTEN 3'-UTR or PTEN 3'-UTR with predicted target site was inserted into pGL3 promoter vector. Cells in the logarithmic growth phase were first seeded into a 96-well plate at a density of 1.5×10^4 cells per well. Subsequently, the cells were cultured in an incubator for 24 hours. After a final concentration of 50 nmol/L miRNA mimics or Non-target Control (NC), 100 ng of target gene 3'UTR double reporter vector or mutant vector were co-transfected for 48 h. Afterward, the medium was aspirated and fresh medium was added at 35 μL/well. Then, the substrate was added to a substrate of 35 μL/well of Luciferase (Promega, Madison, WI, USA), and shaken for 10 min. Finally, the fluorescence value was measured. The experiment was repeated 3 times.

Lactic Dehydrogenase Enzyme (LDH), Malondialdehyde (MDA), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GSH-PX) Detection

Commercial kits for LDH, MDA, SOD and GSH-PX were prepared. Transfected cells were collected, and the expression levels of the above four markers were measured according to the manufacturer's instructions.

Cell Counting Kit-8 (CCK-8) Assay

Pretreated cells were first seeded into 96-well plates at a concentration of 5×10^3/mL, and the total volume per well was 100 μL. 6, 24, 48, and 72 hours after transfection, 10 μL/well of Cell Counting Kit-8 reagent (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, followed by incubation for 1-2 h in the dark. Finally, the absorbance of each well at 450 nm was measured.

Western Blot Assay

After discarding the culture medium, the cells were washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) 3 times. 50 μL of radio-immunoprecipitation assay (RIPA; Beyotime, Shanghai, China) lysate containing phenylmethanesulfonyl fluoride (PMSF; Thermo Fisher Scientific, Waltham, MA, USA) was added to lyse the cells on ice for 30 min. Then, the protein was collected by a spatula. After mixing with 20 μL of 6× loading buffer, extracted protein sample was treated at 100°C for 5 min and then stored at -20°C for subsequent use. The protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred onto membranes. After blocking overnight, the membranes were incubated with primary antibodies. On the next day, the membranes were incubated with corresponding secondary antibodies at room temperature for 2 h. Finally, exposure, development, fixing and filming were performed.

Statistical Analysis

Counting data were expressed by the number of cases. Chi-square test was used to compare the difference between groups. Measurement data were expressed as mean ± standard deviation (mean ±

SD). The *t*-test was used to compare the difference between the two groups. Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analysis. $p<0.05$ was considered statistically significant.

Results

MicroRNA-221 was Highly Expressed in H/R Cells

QRT-PCR assay indicated that the expression level of microRNA-221 in H/R-treated HCM cells was significantly higher than that of the normoxic treated control group (Figure 1A). Then, the microRNA-221 inhibitor was transfected into H/R cells. Subsequent qRT-PCR results showed that microRNA-221 level was markedly decreased (Figure 1B). Next, we detected the levels of myocardial injury markers as well. The results showed that after down-regulation of microRNA-221, the expression levels of myocardial injury markers, including LDH, MDA, and GSH-PX, were remarkably reduced (Figure 1C-1E), whereas SOD level was significantly elevated (Figure 1F).

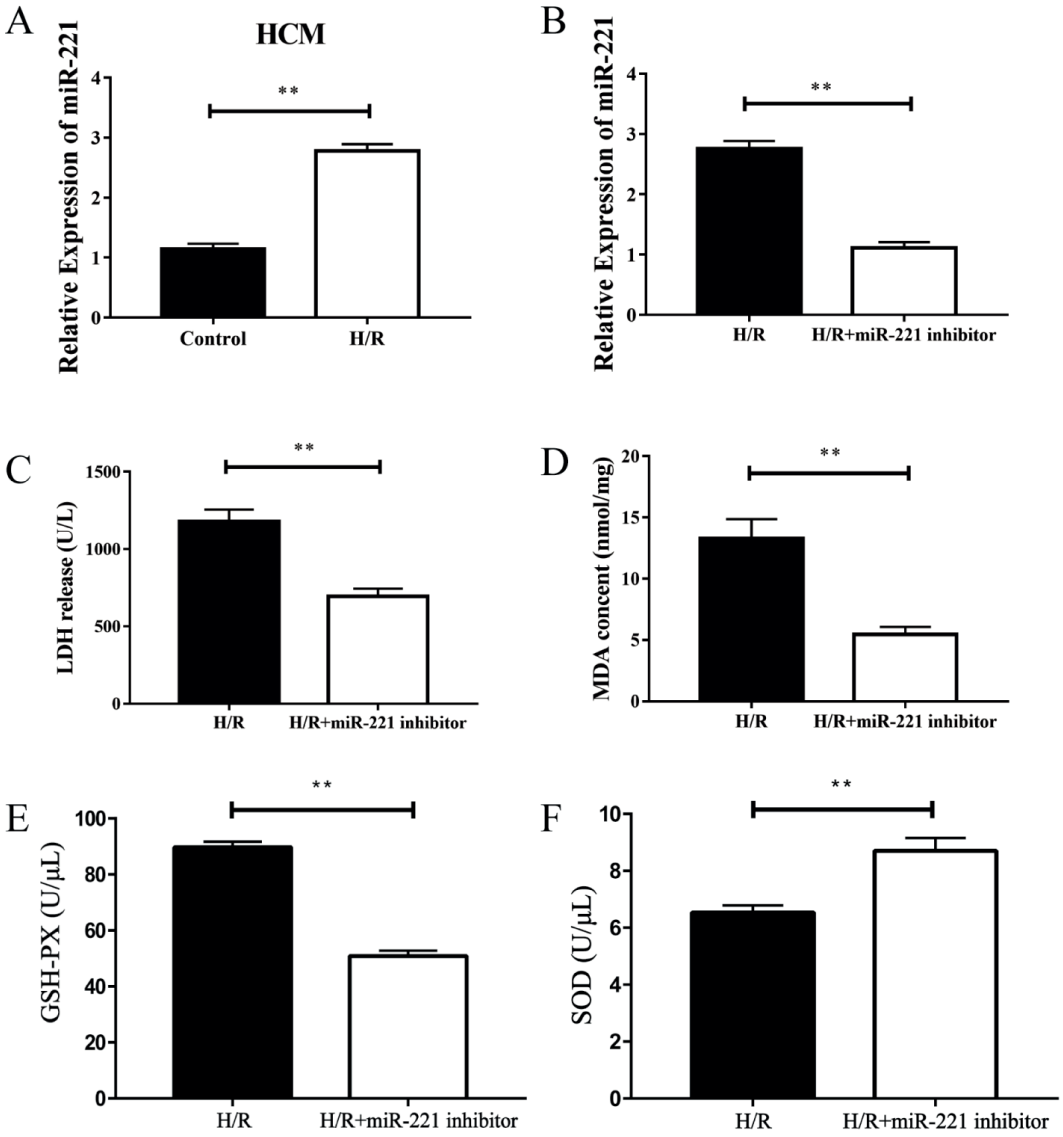

Figure 1. MicroRNA-221 was highly expressed in myocardial H/R cells. ***A,*** MicroRNA-221 was highly expressed in HCM cells deficient in oxygenation. ***B,*** Transfection efficiency of microRNA-221 inhibitor in HCM cells. ***C,*** Down-regulation of microRNA-221 inhibited LDH expression. ***D,*** Down-regulation of microRNA-221 inhibited MDA expression. ***E,*** Down-regulation of microRNA-221 inhibited GSH-PX expression. ***F,*** Down-regulation of microRNA-221 increased SOD expression (**$p<0.01$).

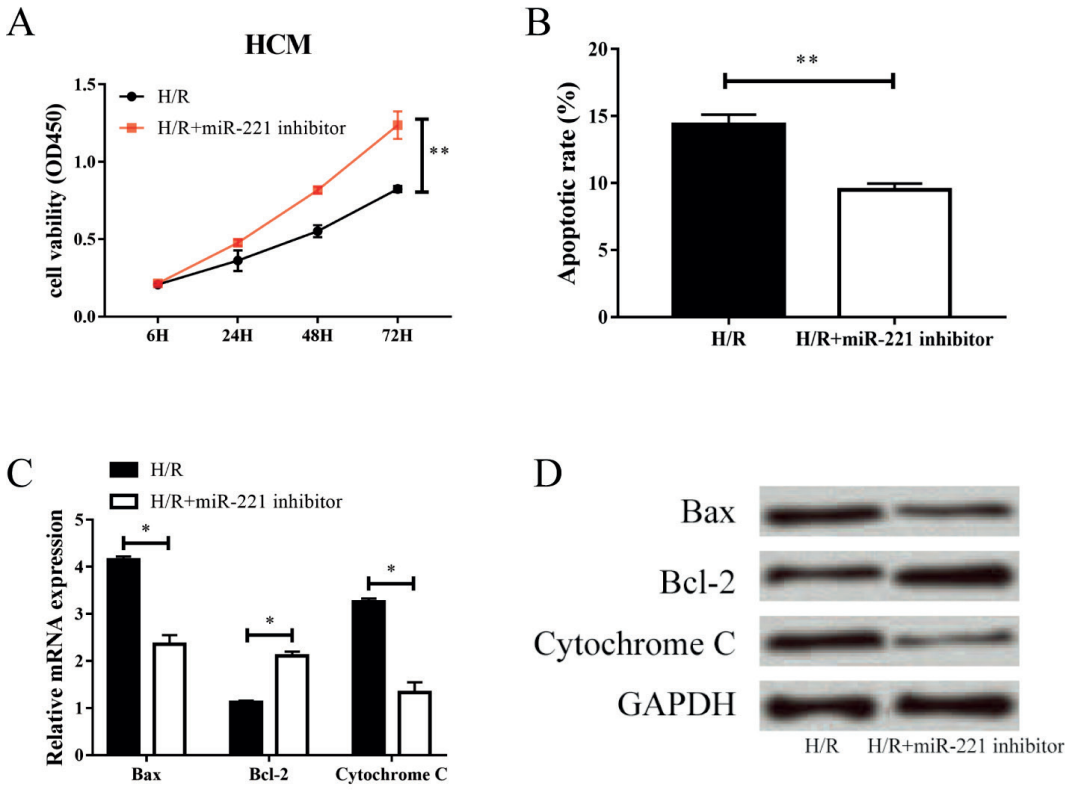

Figure 2. Down-regulation of microRNA-221 inhibited cardiomyocyte apoptosis. ***A,*** CCK-8 results indicated that down-regulation of microRNA-221 could promote cell proliferation. ***B,*** Flow cytometry indicated down-regulation of microRNA-221 could reduce cell apoptosis. ***C,*** QRT-PCR assay indicated down-regulation of microRNA-221 could inhibit the mRNA expression of pro-apoptotic genes, including Bax, and cytochrome C protein, while enhance the expression inhibiting-apoptotic genes such as Bcl-2. ***D,*** Western blot experiment indicated that down-regulation of microRNA-221 could inhibit the protein expression of pro-apoptotic genes, including Bax, and cytochrome C protein, and enhance the expression of inhibiting-apoptotic genes such as Bcl-2 (*p<0.05, **p<0.01).

Downregulation of MicroRNA-221 Inhibited Cardiomyocyte Apoptosis

Subsequently, we investigated the effect of microRNA-221 down-regulation on the proliferation of H/R cells. CCK-8 results indicated that cell proliferation was significantly enhanced after down-regulation of microRNA-221 (Figure 2A). At the same time, flow cytometry demonstrated that down-regulation of microRNA-221 markedly inhibited cell apoptosis (Figure 2B).

To further explore the mechanism of microRNA-221 in regulating cell apoptosis, qRT-PCR was performed after down-regulation of microRNA-221. The results revealed that the expression of pro-apoptotic genes, such as Bax and cytochrome C, was significantly decreased. However, the expression of the inhibitory gene such as Bcl-2 was remarkably increased (Figure 2C). Western blot experiments indicated the same results at the protein level (Figure 2D). Thus, we found that down-regulation of microRNA-221 could significantly promote cardiomyocyte proliferation, whereas inhibiting cell apoptosis.

MicroRNA-221 Could Target PTEN

Bioinformatics analysis was used to predict potential binding targets for microRNA-221 and PTEN (Figure 3A). Meanwhile, PTEN wild and mutant sequences were constructed. Dual-Luciferase reporter assay showed that after transfection of microRNA-221 in HCM cells, the Luciferase was significantly reduced in WT 3'UTR group. However, the Luciferase activity of PTEN-MUT 3'UTR was not conspicuously changed (Figure 3B). At the same time, it was found that microRNA-221 inhibitor could remarkably inhibit microRNA-221 level, whereas up-regulate PTEN expression (Figure 3C). However, the opposite result was observed after transfection of si-PTEN (Figure 3D, 3E). The above findings indicated that

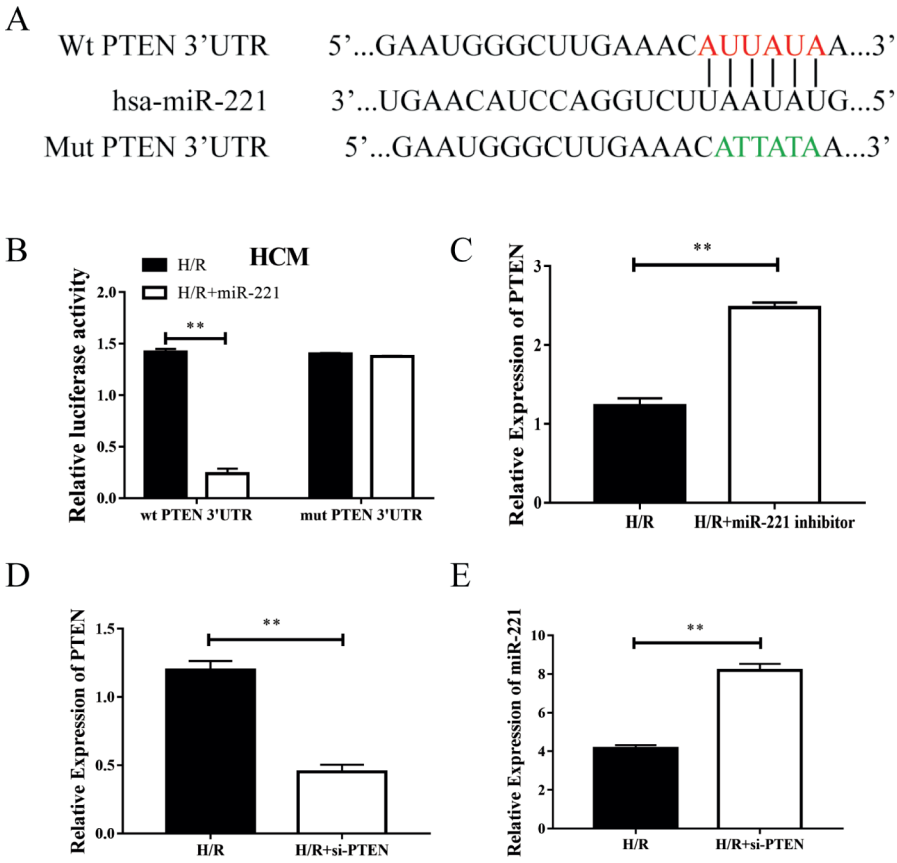

Figure 3. MicroRNA-221 could target bind to PTEN. ***A,*** Bioinformatics was used to predict potential binding sites of microRNA-221 and PTEN. ***B,*** Dual-Luciferase reporter gene experiment showed that microRNA-221 and PTEN could be targeted for binding. ***C,*** PTEN expression level was markedly increased after down-regulation of microRNA-221. ***D,*** Si-PTEN transfection efficiency. ***E,*** Decreased expression of microRNA-221 after down-regulation of PTEN (**p<0.01).

microRNA-221 could target and bind to PTEN, eventually regulating its expression.

MicroRNA-221 Promoted Cardiomyocyte Apoptosis by Downregulating PTEN

Through bioinformatics prediction, we found that microRNA-221 could regulate the expression of PTEN. QRT-PCR analysis showed that the expression level of PTEN in H/R cells was significantly lower than that of the normoxic treatment group (Figure 4A). We conspicuously diminished cell proliferation after down-regulating PTEN in H/R cells (Figure 4B). Flow cytometry revealed that down-regulation of PTEN remarkably promoted the apoptosis of cardiomyocytes (Figure 4C). To further explore the relationship between PTEN and apoptosis, qRT-PCR was performed to detect the expression levels of pro-apoptotic genes, including Bax and cytochrome C, after PTEN down-regulation. The results demonstrated that the expression levels of the two genes were also significantly enhanced. However, the expression of Bcl-2 was markedly down-regulated (Figure 4D). The same findings were obtained by Western blot analysis at protein levels (Figure 4E); they indicated that the overexpression of microRNA-221 might promote cardiomyocyte apoptosis by down-regulating PTEN.

Discussion

Ischemic heart disease, such as acute myocardial infarction, is currently treated by thrombolysis, intervention and bypass surgery. It can restore the blood flow of ischemic myocardium and reduce the range of myocardial infarction as soon as possible. However, studies have found that, after myocardial ischemia and reperfusion, the production of reactive oxygen species and activa-

tion of neutrophils may happen. This can eventually lead to the occurrence of inflammation, myocardial cell damage and even cell apoptosis[16]. Therefore, cardiomyocyte apoptosis is closely related to myocardial ischemia-reperfusion, which is a landmark event during myocardial ischemia-reperfusion[17]. How to reduce the number of apoptotic cells in the heart, protect the structure and function of myocardial cells, and reduce the area of the dangerous area around infarction have become hot topics in the field of cardiovascular research in recent years[18]. Previous studies have shown that the mechanism of myocardial apoptosis induced by myocardial ischemia-reperfusion is very complicated. The process requires complete regulation of a variety of apoptosis-related genes. Bcl-2 and Bax are considered as an important group of apoptosis-regulating genes[19]. Bax promotes cell apoptosis, while Bcl-2 inhibits apoptosis. The equilibrium state of the two genes directly affects the regulation of cell apoptosis. Therefore, the balance between the content and

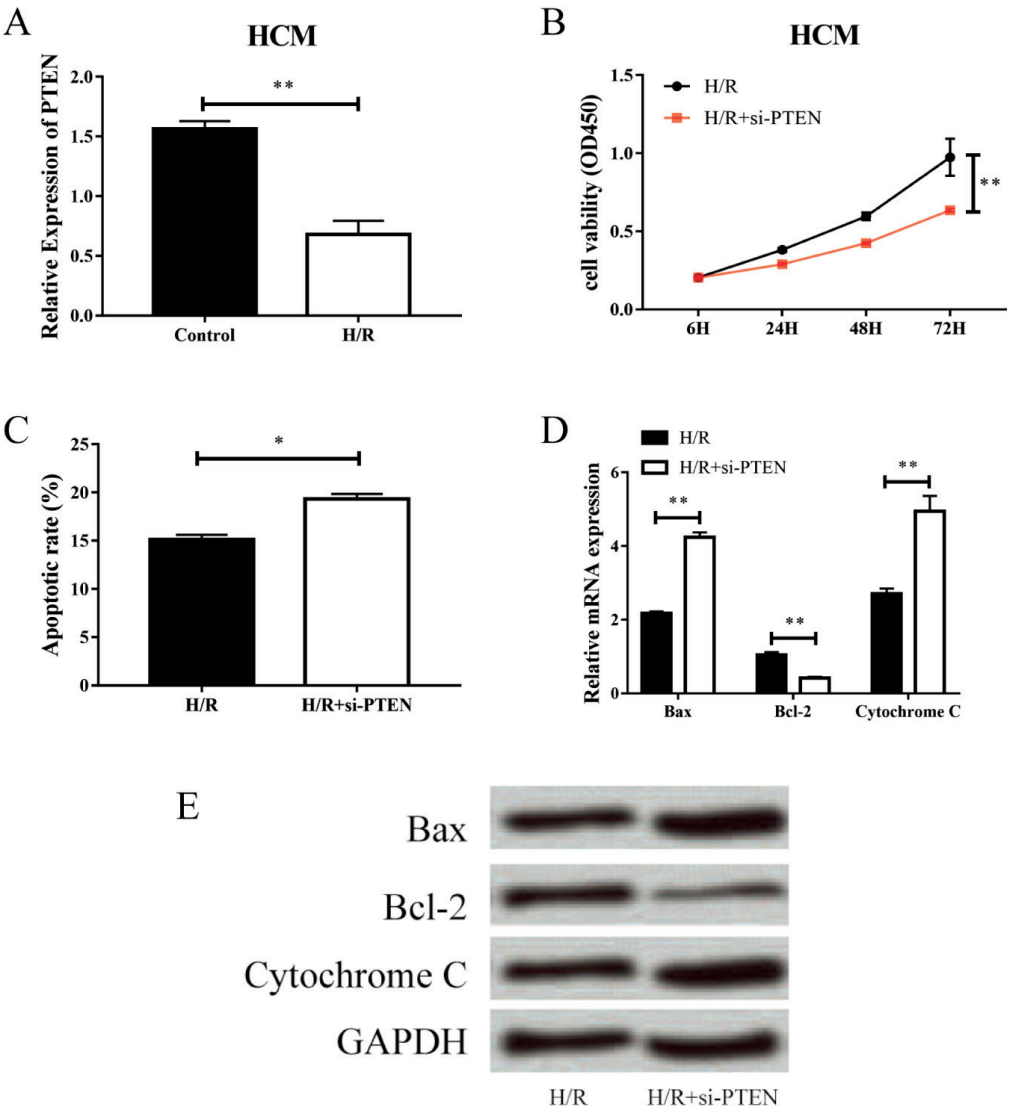

Figure 4. MicroRNA-221 promoted cardiomyocyte apoptosis by down-regulating PTEN. ***A,*** PTEN was lowly expressed in HCM cells deficient in re-oxygenation. ***B,*** CCK-8 results showed that cell proliferation was significantly weakened after down-regulation of PTEN. ***C,*** Flow cytometry result showed that down-regulation of PTEN reduced cell apoptosis. ***D,*** QRT-PCR analysis showed that down-regulation of PTEN could enhance the mRNA expression of pro-apoptotic genes including Bax, and cytochrome C protein, while inhibit the expression of inhibiting-apoptotic genes such as Bcl-2. ***E,*** Western blot experiment showed that down-regulation of PTEN could enhance the protein expression of pro-apoptotic genes including Bax, and cytochrome C protein, whereas inhibit the expression of inhibiting-apoptotic genes such as Bcl-2 (*p<0.05, **p<0.01).

function of both Bcl-2 and Bax in cells is an important mechanism of cell death or survival.

PTEN can dephosphorylate phosphatidylinositol triphosphate (PIP3) into phosphatidylinositol diphosphate (PIP2), which negatively regulates the PI3K/AKT signaling pathway. Meanwhile, it plays an important role in cell apoptosis. Studies have shown that microRNA-221 participates in the development of various tumor cells by regulating the PTEN/AKT signaling pathway along with the expressions of Bax and Bcl-2[20,21]. PTEN is known as an attractive target for myocardial protection. However, only a few studies[22,23] have evaluated the role of PTEN in ischemia-reperfusion or hypoxia models. Difficulty in studying this phosphatase may be related to the lack of highly specific activators or inhibitors. In addition, studies[24] have also indicated that PTEN protects the heart from ischemia-reperfusion injury by up-regulating PI3K/Akt/endothelial nitric oxide synthase/ERK pathway, which is one kind of pro-apoptotic pathway.

In this work, we found that the expression level of microRNA-221 in H/R treated cells was significantly up-regulated when compared that of the normoxic treated group. After microRNA-221 down-regulation, the expression levels of myocardial injury markers, including LDH, MDA and GSH-PX in H/R cells, were markedly decreased, while SOD level was increased. *In vitro* researches showed that the proliferation ability and apoptosis of cells were remarkably decreased after down-regulation of microRNA-221. At the same time, down-regulation of microRNA-221 could significantly reduce the mRNA and protein expression levels of apoptosis-promoting genes, including Bax and cytochrome C. However, microRNA-221 down-regulation could enhance the expression levels of apoptosis inhibitory genes such as Bcl-2. In addition, the Dual-Luciferase reporter gene assay showed that microRNA-221 could target and bind to PTEN and inhibit its expression. In H/R cells, we found that the expression level of PTEN was significantly lower than that of the normoxic control group. Further studies indicated that down-regulation of PTEN markedly decreased cell proliferation and apoptosis, and increased the mRNA and protein expression levels of apoptosis-promoting genes including Bax and cytochrome C. However, down-regulation of PTEN could remarkably reduce the expression levels of apoptosis-inhibiting genes such as Bcl-2. Therefore, we believed that microRNA-221 could promote myocardial apoptosis induced by myocardial ischemia and reperfusion. The possible underlying mechanism might be related to the down-regulation of PTEN expression.

Conclusions

We found that microRNA-221 could promote myocardial apoptosis induced by myocardial ischemia-reperfusion by down-regulating PTEN expression. Our findings indicated that microRNA-221 might become a potential therapeutic target for myocardial ischemia-reperfusion injury.

Conflict of interest

The authors declare no conflicts of interest.

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