The expression of microRNA-23a regulates acute myocardial infarction in patients and *in vitro* through targeting PTEN

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Abstract. Cardiovascular disease is responsible for one of the highest rates of fatality worldwide. The present study investigated the presence and influence of microRNA (miRNA)-23a in the regulation of acute myocardial infarction (AMI). A total of 6 patients with AMI and 6 normal volunteers without myocardial disease were included, and blood samples were taken to analyze the expression of miRNA-23a by reverse transcription-quantitative polymerase chain reaction. miRNA-23a expression in patients with AMI was downregulated compared with the normal group. In H9C2 cells treated with H_2O_2, upregulation of miRNA-23a expression increased the superoxide dismutase, glutathione and catalase activity levels, and suppressed the malonaldehyde activity level, as determined by ELISA. Western blot analysis and a caspase-3 substrate assay demonstrated that upregulation of miRNA-23a expression suppressed the Bcl-2-associated X (Bax)/Bcl-2 protein expression ratio, caspase-3 activity level and tumor suppressor p53 (p53) protein expression in H_2O_2-induced H9C2 cells. Furthermore, downregulation of phosphatase and tensin homolog (PTEN), by the PTEN inhibitor bpV(HOpic), increased miRNA-23a expression and suppressed the Bax/Bcl-2 protein expression ratio, caspase-3 activity level and p53 protein expression in H_2O_2-induced H9C2 cells. Therefore, the results of the present study indicate that the expression of miRNA-23a may regulate AMI through targeting PTEN in patients and *in vitro*, and PTEN/miRNA-23a may therefore be potential targets for the clinical treatment of AMI.

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Introduction

Cardiovascular disease is one of the diseases with the highest fatality rates worldwide. In the United States, ~1/6th of the annual death toll is associated with coronary heart disease and associated diseases with myocardial injury (1). Acute myocardial infarction (AMI) results in ventricular remodeling that causes the deterioration of heart function leading to heart failure (2). Myocardial fibrosis leads to decreased myocardial contractility and subsequent ventricular systolic dysfunction (3).

Myocardial remodeling, including the remodeling of myocardial cells and the extracellular matrix (ECM), is key for the transformation process of heart disease to heart failure (4). Previously, it was hypothesized that the function of the ECM is constant and is primarily responsible for connection, support and protection (5). Recently, a study demonstrated that ECM is in an active state and is actively involved in intracellular and extracellular signaling and the maintenance of cardiac structure and function (5). Therefore, ECM remodeling serves an important role in the initiation and development of heart failure.

AMI is a cardiovascular disease with serious implications for human health. It may lead to heart failure and a number of other conditions with poor prognoses (6). Myocardial fibrosis is an important pathophysiological alteration following AMI. It is activated as a result of multiple fibrosis-associated factors and leads to interstitial cell hyperplasia (7). Myocardial fibrosis increases cardiac stiffness, decreases diastolic systolic function and alters the normal structure of cardiac electrophysiology, which leads to arrhythmia and potentially even sudden death (8).

Phosphatase and tensin homolog (PTEN) is a tumor suppressor that was discovered in 1997. The genes that encode PTEN are located at 10q23.3 and encoded by a dual specificity phosphatase consisting of 403 amino acids (9). PTEN is a dual specificity phosphatase with lipid phosphatase and protein phosphatase activity (9). PTEN enables the secondary messenger produced by phosphatidylinositol 3-kinase (PI3K) to phosphorylate and prevent protein kinase B (Akt) from being activated and inhibits its downstream biological functions (10). It has been reported that PTEN is associated with ischemic heart disease (11). Blocking the

PTEN signaling pathway confirmed that PTEN influences myocardial ischemic injury events and reduces the death of myocardial cells (11). However, studies on myocardial ischemic injury in which one or several genes that regulate cardiomyocyte apoptosis through PTEN are investigated have not previously been performed (11).

MicroRNAs (miRNAs) are a group of non-coding RNAs that are endogenous, short (~22 bp) (12) and are highly conserved in sequence between species. Currently, there are ~1,400 types of miRNAs in humans and novel miRNA are frequently identified. Each type of miRNA serves an important role in the transcription processes in the body by regulating the expression of various target genes (13). miRNA influences cells in numerous ways, including the regulation of cellular apoptosis and necrosis, and these two mechanisms are of great importance in ventricular remodeling during the developmental phase of AMI. The results of the present study help to elucidate the presence and role of miRNA-23a in the regulation of AMI.

Materials and methods

Patient selection and blood collection. The present study was approved by the Regional Ethics Committee of Beijing Chaoyang Hospital (Beijing, China) and all patients involved gave their written informed consent. A total of 6 patients with AMI (aged 57-66 years, male) and 6 normal volunteers (aged 23-28 years, male) without myocardial disease were recruited between July 2015 and August 2015 from the Department of Internal Medicine, Beijing Chaoyang Hospital. Between 8:00 and 10:00 a.m., blood samples (5 ml) were collected and centrifuged at 2,000 x g for 10 min at 4˚C to be stored at -80˚C.

Cell culture. The H9C2 cell line was purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime Institute of Biotechnology, Haimen, China) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cell transfection. The miRNA-23a mimic (5'-ggccggctg gggttcctggg-3') and the negative controls (5'-CCCCCCCCC C-3') were purchased from MyGenostics, Inc., (Beijing, China). Following 6 h of incubation at 37˚C with Dulbecco's modified Eagle medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), cells ($1x10^6$) were transfected with miRNA-23a mimic (50 nM) and negative controls (50 nM) for 48 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, 4 h following transfection of H9C2 cells with miRNA-23a, the cells were treated with 600 μM H_2O_2 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with or without the PTEN inhibitor, bpV(HOpic) (1 μM; cat. no. sc-33022; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), for 2 h at 37˚C. Cells that were transfected with negative controls received treatment with H_2O_2 only.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from blood samples or cell samples using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (0.5-1 μg) was reverse transcribed into cDNA using an iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 37˚C for 60 min and 85˚C for 5 min. qPCR was performed using Takara SYBR Premix Ex Taq™ (Tli RNase H Plus; Takara Bio, Inc., Otsu, Japan) on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with the following thermocycling conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 25 sec, 60˚C for 30 sec and 72˚C for 30 sec. The primers used for qPCR were as follows: miR-23a forward, 5'-GGGGGG GGATCACATTGCCA-3' and reverse, 5'-CAGTGCAGGGTC CGAGGT-3'; and U6 forward, 5'-GCTTCGGCAGCACAT ATACTAAAAT and reverse, 5'-CGCTTCACGAATTTG CGTGTCAT-3'. The relative expression level of miRNA-23a was calculated by the $2^{-\Delta\Delta Cq}$ method (14).

Measurement of oxidative stress. H9C2 cells ($1x10^5$ cells) transfected with miRNA-23a or negative controls were treated with 600 μM H_2O_2 with or without 1 μM bpV(HOpic) for 2 h. Subsequently, H9C2 cells were lysed in radioimmunoprecipitation buffer (RIPA; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) containing phenylmethanesulfonyl fluoride (PMSF; 1:100; Nanjing KeyGen Biotech Co., Ltd.). Total proteins were quantified with a bicinchoninic assay (BCA) protein kit (Beyotime Institute of Biotechnology). Total protein (10-20 μg) was incubated with superoxide dismutase (SOD, cat. no. A001-3), glutathione (GSH; cat. no. A006-2), catalase (CAT; cat. no. A007-1-1) and malondialdehyde (MDA, cat. no. A003-1) ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Absorbance was measured at 450 nm.

Measurement of caspase-3 activity level. H9C2 cells ($1x10^5$ cells) transfected with miRNA-23a or negative controls were treated with 600 μM H_2O_2 with or without 1 μM bpV(HOpic) for 2 h. Subsequently, H9C2 cells were lysed in RIPA buffer containing PMSF (1:100). Total proteins were quantified with a BCA protein assay kit. A total of 10 μg total protein was incubated with the caspase-3 substrate Ac-DEVD-pNA (2 mM; Beyotime Institute of Biotechnology) for 2 h at 37˚C. Absorbance was measured at 405 nm.

Western blot analysis. H9C2 cells ($1x10^5$ cells) transfected with miRNA-23a or negative controls were treated with 600 μM H_2O_2 with or without 1 μM bpV(HOpic) for 2 h. Subsequently, H9C2 cells were lysed in RIPA buffer containing PMSF (1:100). Total proteins were quantified with a BCA protein assay kit. A total of 50-60 μg total protein was subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% non-fat milk for 1 h at 37˚C and then probed with primary antibodies against Bcl-2-associated X (Bax; cat. no. A2211; 1:2,000; ABclonal Biotech Co., Ltd., Woburn, MA, USA), Bcl-2 (cat. no. A0208; 1:2,000; ABclonal Biotech Co., Ltd.), tumor suppressor p53 (p53; cat. no. AP0464; 1:1,000; ABclonal Biotech Co., Ltd.) and GAPDH (cat. no. A0080; 1:2,000; ABclonal Biotech Co., Ltd.) overnight at 4˚C.

Following incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1;10,000; cat. no. HSA0003; Shanghai Bioon Biotechnology Co., Ltd., Shanghai, China) and anti-mouse secondary antibodies (1:5,000; cat. no. sc-2020; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, Clarity Max™ Western ECL Substrate (cat. no. 1705062; Bio-Rad Laboratories, Inc.) was used for visualization. Protein bands were visualized using Image-ProPlus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. The results are presented as the mean ± standard deviation. All data were analyzed with an independent-samples Student t-test or one-way analysis of variance test followed by Bonferroni's tests using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miRNA-23a in patients with AMI. miRNA-23a expression was analyzed in patients with AMI and healthy volunteers. The results of RT-qPCR demonstrated that miRNA-23a expression in patients with AMI was significantly downregulated compared with the healthy volunteer group ($P<0.01$; Fig. 1).

Expression of miRNA-23a in vitro in AMI. In addition, the miRNA-23a mimic was transfected into H9C2 cells, which promoted miRNA-23a expression in H9C2 cells. Transfection of the miRNA-23a mimic in H_2O_2-treated H9C2 cells significantly increased the expression of miRNA-23a, compared with the negative control group ($P<0.01$; Fig. 2).

Effect of miRNA-23a expression on oxidative stress in vitro in AMI. To investigate the functional effect of miRNA-23a in H9C2 cells treated with H_2O_2, ELISAs were conducted to analyze levels of oxidative stress markers. There was a significant increase in the activity of SOD, GSH and CAT, and a decrease in the activity of MDA, in miRNA-23a-overexpressing H9C2 cells treated with H_2O_2 compared with the negative control group ($P<0.01$; Fig. 3).

Effect of miRNA-23a expression on apoptosis in vitro in AMI. To investigate the functional effect of miRNA-23a on apoptosis in H_2O_2-treated H9C2 cells, a commercial ELISA kit was employed to analyze caspase-3 activity. As demonstrated in Fig. 4A, in the miRNA-23a-overexpressing H9C2 cells treated with H_2O_2, the caspase-3 activity was significantly decreased compared with the negative control group. In addition, the Bax/Bcl-2 and p53 protein expression was significantly decreased in miRNA-23a-overexpressing H9C2 cells treated with H_2O_2 compared with the negative control group ($P<0.01$; Fig. 4B-D).

PTEN inhibitor effect on the in vitro expression of miRNA-23a in AMI. In order to investigate the association between miRNA-23a and PTEN expression in AMI, the PTEN inhibitor bpV(HOpic) was added to miRNA-23a-overexpressing H9C2 cells. In the miRNA-23a-overexpressing H9C2 cells treated with H_2O_2, the PTEN inhibitor significantly increased miRNA-23a expression, compared with the miRNA-23a-overexpressing group alone ($P<0.01$; Fig. 5).

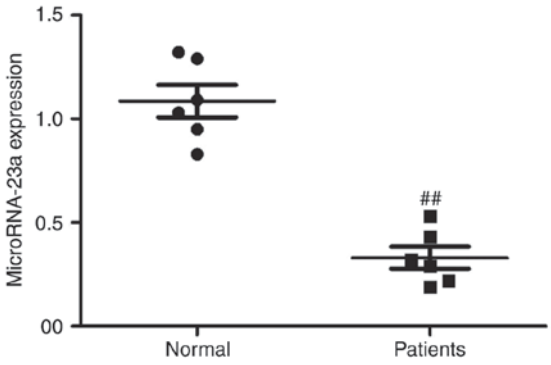

Figure 1. Expression of miRNA-23a in patients with acute myocardial infarction. ##$P<0.01$ vs. normal group. miRNA, microRNA.

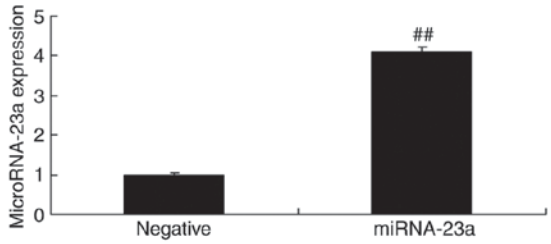

Figure 2. Expression of miRNA-23a in an *in vitro* model of acute myocardial infarction. ##$P<0.01$ vs. negative control group. miRNA, microRNA.

Effect of PTEN inhibitor on in vitro oxidative stress in AMI. In order to investigate the effect of PTEN on oxidative stress *in vitro* in AMI, miRNA-23a-overexpressing H9C2 cells were treated with H_2O_2. There was a significant increase in the activities of SOD, GSH and CAT, and a decrease of MDA content, compared with miRNA-23a-overexpressing H9C2 cells treated with H_2O_2 alone ($P<0.01$; Fig. 6).

Effect of PTEN inhibitor on apoptosis in AMI. The effect of PTEN on *in vitro* on apoptosis in AMI was investigated. As demonstrated in Fig. 7, the downregulation of PTEN significantly suppressed Bax/Bcl-2 and p53 protein expression, and caspase-3 activity level, in miRNA-23a-overexpressing H9C2 cells treated with H_2O_2, compared with the miRNA-23a-overexpressing H9C2 cells treated with H_2O_2 alone ($P<0.01$).

Discussion

With rapid socioeconomic development and improvements in living standards, and the alteration of modern lifestyles, the incidence of cardiovascular and cerebrovascular disease is increasing (15). The high morbidity rate and high case-fatality rate have serious effects on the physical and mental health of individuals (16). In China, the case-fatality rate of cardiocerebral vascular diseases is the second largest following malignant tumors (17). Solutions to the relevant problems are urgently required, such as methods that allow more in-depth research into the pathogenesis of cardiovascular and cerebrovascular diseases, and the development of more effective and safer clinical drugs (18).

Apoptosis is a form of programmed cell death. In the acute period of myocardial infarction, apoptosis is involved in

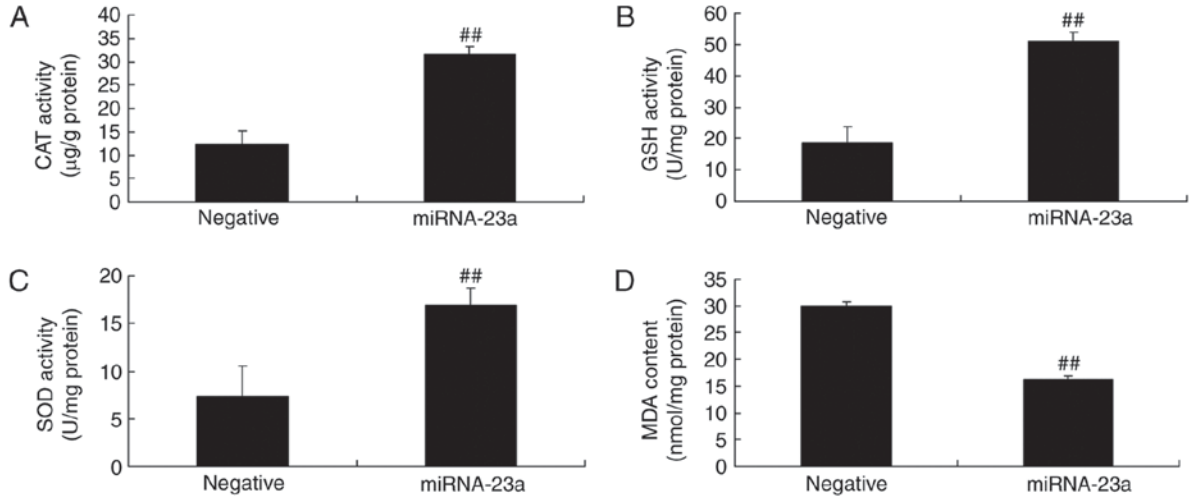

Figure 3. Effect of miRNA-23a expression on oxidative stress in an *in vitro* model of AMI. The effect of the expression of miRNA-23a on (A) CAT, (B) GSH and (C) SOD activity levels, and (D) MDA levels, in an *in vitro* model of AMI. $^{\#\#}$P<0.01 vs. negative control group. miRNA, microRNA; AMI, acute myocardial infarction; CAT, catalase; GSH, glutathione; SOD, superoxide dismutase; MDA, malondialdehyde.

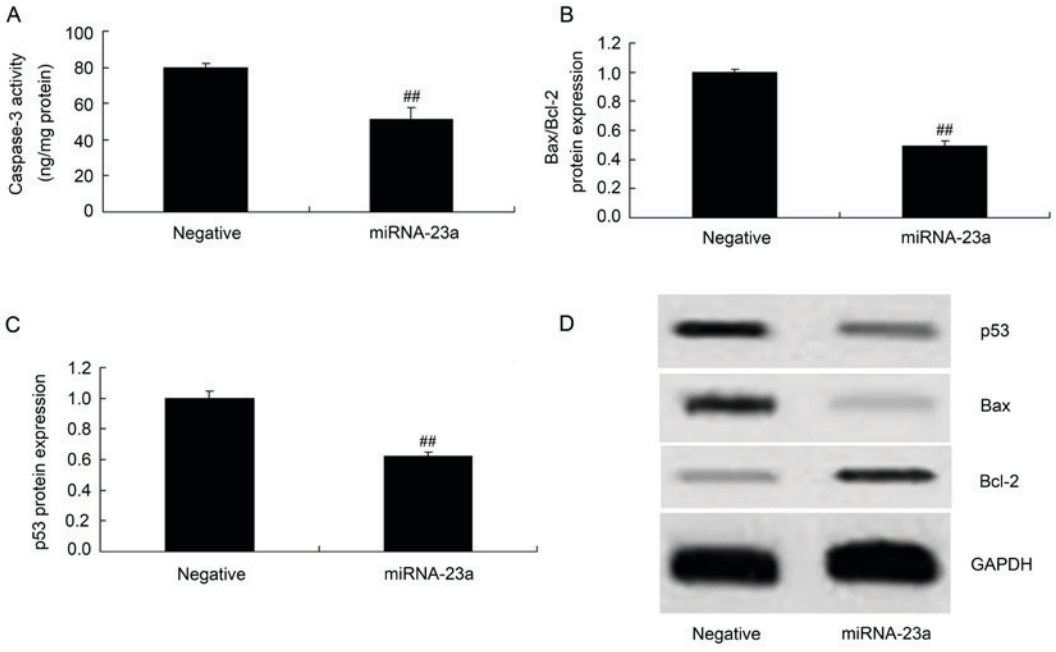

Figure 4. Effect of miRNA-23a expression on apoptosis in an *in vitro* model of AMI. (A) Quantitative analysis of the effect of miRNA-23a expression on caspase-3 activity in an *in vitro* model of AMI. Densitometric and statistical analysis of (B) Bax/Bcl-2 protein expression ratio and (C) p53 protein expression. (D) Representative western blot images for Bax, Bcl-2 and p53 protein expression in an *in vitro* model of AMI. $^{\#\#}$P<0.01 vs. negative control group. miRNA, microRNA; AMI, acute myocardial infarction; Bax, Bcl-2-associated X; p53, tumor suppressor p53.

the death of myocardial cells. It is similar to that myocardial has lost its improvement process in subacute or stale stage of myocardial infarction. miRNAs influence the regulation of cellular apoptosis and necrosis, and these two mechanisms are essential for the ventricular remodeling during the developmental phase of AMI (12). It has been demonstrated that there are numerous alterations in the expression of miRNAs in blood samples during the acute stage of AMI (19). If the association between miRNA expression and AMI could be elucidated, molecular markers may be identified for early clinical diagnosis of AMI to improve the survival rate of patients with AMI (20). Therefore, a number of studies in

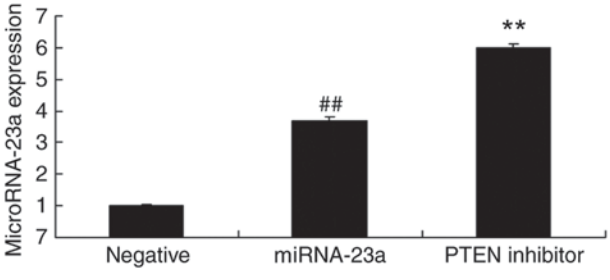

Figure 5. PTEN inhibitor effects on the miRNA-23a expression in an *in vitro* model of acute myocardial infarction. $^{\#\#}$P<0.01 vs. negative control group; **P<0.01 vs. miRNA-23a group. PTEN, phosphatase and tensin homolog; miRNA, microRNA.

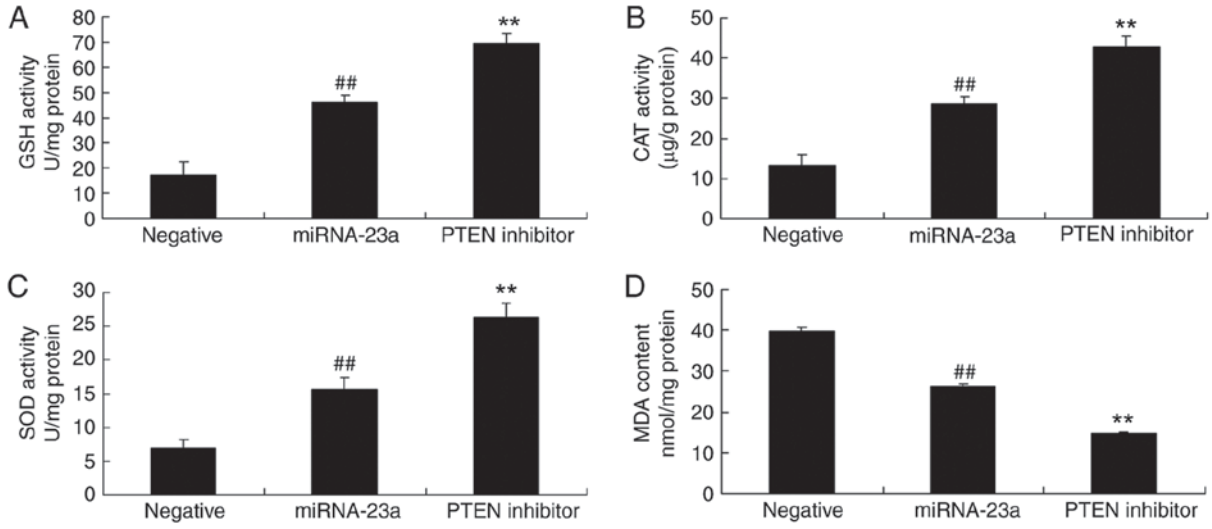

Figure 6. Effects of a PTEN inhibitor on oxidative stress in an *in vitro* model of AMI. Effects of a PTEN inhibitor on (A) GSH, (B) CAT and (C) SOD activity, and (D) MDA levels, in an *in vitro* model of AMI. $^{\#\#}$P<0.01 vs. negative control group; **P<0.01 vs. miRNA-23a group. PTEN, phosphatase and tensin homolog; AMI, acute myocardial infarction; GSH, glutathione; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; miRNA, microRNA.

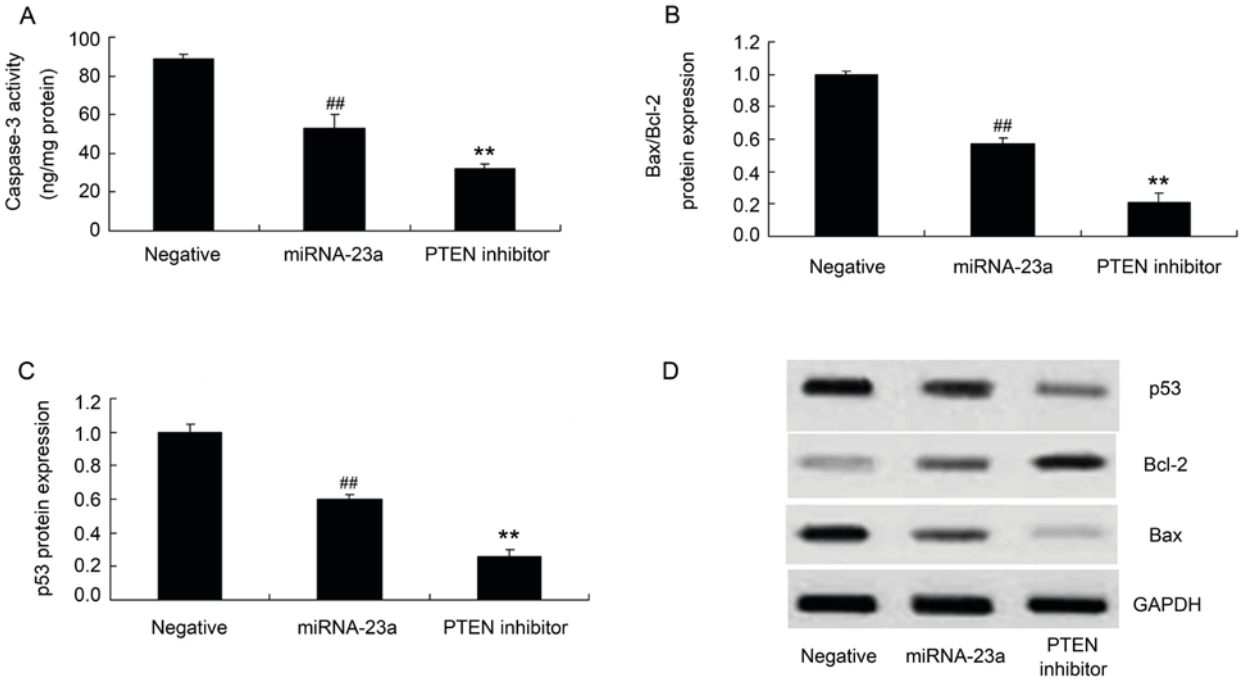

Figure 7. Effects of a PTEN inhibitor on apoptosis in an *in vitro* model of AMI. Effects of a PTEN inhibitor on (A) caspase-3 activity, (B) Bax/Bcl-2 protein expression ratio and (C) p53 protein expression. (D) Representative western blot images for Bax, Bcl-2 and p53 protein expression in an *in vitro* model of AMI. $^{\#\#}$P<0.01 vs. negative control group; **P<0.01 vs. miRNA-23a group. PTEN, phosphatase and tensin homolog; AMI, acute myocardial infarction; Bax, Bcl-2-associated X; p53, tumor suppressor p53; miRNA, microRNA.

China and worldwide have been initiated using clinical and animal experimental models. In the present study, miRNA-23a expression in patients with AMI was demonstrated to be downregulated compared with the healthy volunteer group. Mao *et al* (21) identified miRNA-23a as being involved in tumor necrosis factor-α-induced protection of bone marrow-derived mesenchymal stem cell apoptosis through regulating caspase-7 and myocardial infarction.

A previous study demonstrated that oxidative stress is a common mechanism of various factors that cause cardiovascular injury (22). During the pathological process of a number of cardiovascular diseases, including atherosclerosis, hypertension, ischemic heart disease and hyperlipidemia, excessive oxygen free radical production and suppression of the antioxidant defense mechanism for managing free radicals has been observed (22). Previously, attention has focused on the influence of reactive oxygen species (including oxygen free radicals) on cardiac and vascular function. White blood cells produce reactive oxygen species, which cause vascular injury in AMI (23). White blood cells have the ability to release high

concentrations of reactive oxygen species, which kill bacteria and result in vascular injury (8). In addition, AMI also leads to the production of reactive oxygen species, therefore causing tissue damage during reperfusion, including in the heart, brain and liver. It has been reported that reactive oxygen species are essential not only in pathological processes, but also in normal signaling pathways and physiological mechanisms (24). In accordance with this, the present study demonstrated that overexpression of miRNA-23a significantly increased the activity of SOD, GSH and CAT, and inhibited MDA levels, in H_2O_2-treated H9C2 cells. Zhao *et al* (25) demonstrated that miRNA-23a-3p attenuated injury caused by oxidative stress in focal cerebral ischemia-reperfusion mice.

miRNA-23a-3p inhibits the infarction factors that kill the myocardial cells of rats, including the expression of p53, Bax and tumor necrosis factor receptor superfamily member 6 (Fas). Cytochrome C is released and forms an apoptotic body by combining with caspase-9 (26). Subsequently, apoptosis is induced in cells with activated caspase-3 through downstream signaling pathways (27). Studies have demonstrated that caspase-3 and caspase-9 proteases effectively inhibited the apoptosis of cardiac muscle cells by decreasing the activity and expression of caspase-3/9 (28). The Bcl-2 family is reported to affect the release of cytochrome C through mitochondrial channels, inhibit the release of Bax and inhibit the activation of cytochrome C-induced apoptosis. Therefore, Bcl-2 acts as an inhibitor of apoptosis (26).

Fluctuations in the intracellular content of Bcl-2 can induce a pro-apoptotic signaling cascade (29). Bcl-2 family members are among the most important membrane protein molecules in apoptosis and control cell apoptosis by internal and external signals in different cells (29). A number of studies have demonstrated that certain genes promote apoptosis (such as Bax, p53 and Fas), while other genes are able to inhibit apoptosis (such as Bcl-2) (30). The *in vivo* ratio between proapoptotic proteins and inhibitory proteins is key for determining the fate of cells (29). For instance, when Bcl-2 protein expression is higher than Bax protein expression, cell survival is promoted. By contrast, when Bax protein levels are higher compared with Bcl-2 levels, apoptosis is accelerated (26). Therefore, the results of the present study demonstrated that overexpression of miRNA-23a decreased caspase-3 activity, Bax/Bcl-2 ratio and p53 protein expression in H9C2 cells treated with H_2O_2. Wang *et al* (31) revealed that berberine induced tumor apoptosis through upregulation of p53 by miRNA-23a in hepatocellular carcinoma. Wang *et al* (32) also reported that glucagon like peptide-1 reduced apoptosis and Bax expression by downregulating miRNA-23a.

PTEN is a non-secreted protein inherently expressed in cells. It also exists in the nucleus, cytoplasm and membrane (10). It is involved in multiple signal transduction pathways within a cell, including those associated with cell division, proliferation, differentiation, migration and extracellular matrix metabolism, and other physiological and pathological processes (9). In the present study, the PTEN inhibitor was observed to increase miRNA-23a expression in H9C2 cells treated with H_2O_2. Tian *et al* (33) demonstrated that miRNA-23a enhanced migration and invasion in osteosarcoma cells through downregulation of PTEN expression.

PTEN also serves an important role in growth (9). In addition, PTEN regulates developmental malformations and abnormal differentiation in the third germ layer (34). Investigation of the effect of PTEN on heart growth and function in adult mice with a specific knockout of PTEN in cardiac muscle cells demonstrated that muscular hypertrophy caused by external pressure may be inhibited by knocking out PTEN, which therefore protects the heart functions (35). In the present study, the downregulation of PTEN was observed to suppress p53 protein expression in miRNA-23a-overexpressing H9C2 cells treated with H_2O_2.

In summary, the present study has confirmed that the expression of miRNA-23a may regulate AMI-induced apoptosis in patients with AMI and an *in vitro* model, which may occur via inhibition of caspase-3, Bax/Bcl-2 and p53 expression through targeting PTEN. The results of the present study indicate that PTEN/miRNA-23a may be potential therapeutic targets for treating AMI-induced apoptosis in a clinical setting.

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Availability of data and materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QS designed the study; SL and JR performed the experiments; QS analyzed the data; QS wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Regional Ethics Committee of Beijing Chaoyang Hospital (Beijing, China) and all patients involved provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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