

Expression of miRNA-214 in the sera of elderly patients with acute myocardial infarction and its effect on cardiomyocyte apoptosis

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Abstract. The aim of the present study was to investigate the expression of microRNA (miRNA/miR)-214 in the sera of elderly patients with acute myocardial infarction (AMI) and the mechanism of how its expression affects cardiomyocyte apoptosis in these patients. The expression levels of miRNA-214 in elderly patients with AMI, unstable angina (UA) and healthy elderly subjects were detected by reverse transcription-quantitative polymerase chain reaction, and the correlation between the relative expressions of sera miRNA-214 and myocardial enzymes in elderly patients with AMI was examined by Pearson's analysis. Human cardiomyocyte (HCM) cell lines with a high expression miRNA-214 were established. The apoptotic rates of the different groups of cells were detected by flow cytometry and TUNEL assay. The expression of miRNA-214 target genes in the different groups of cells was detected by western blot assay. The relative expression levels of sera miR-214 in elderly AMI patients, UA patients and healthy subjects (as determined by physical examination) were 15.79 ± 4.66 , 4.60 ± 2.51 and 2.07 ± 0.99 , respectively. The differences between each group were statistically significant ($P < 0.05$). The relative expression of sera miRNA-214 in elderly AMI patients was positively correlated with sera aspartate aminotransferase ($r = 0.361$, $P = 0.0174$), lactate dehydrogenase ($r = 0.425$, $P = 0.0045$), creatine kinase-MB ($r = 0.835$, $P < 0.001$) and cardiac troponin ($r = 0.770$, $P < 0.001$). When compared with normal HCMs, the expressions of p53-upregulated modulator of apoptosis (PUMA), phosphatase and tensin homolog (PTEN), B-cell lymphoma-2-associated X protein (Bax) and caspase 7 proteins was decreased in HCMs overexpressing miRNA-214 following H_2O_2 induction, and the rate of apoptosis decreased by 63.64, 21.95, 46.67 and 50.05%, respectively. miRNA-214 was highly expressed in the sera of elderly patients with AMI,

which may inhibit myocardial cell apoptosis by inhibiting the expression of miR-214 target genes including *PUMA*, *PTEN*, *Bax* and *caspase 7*.

Introduction

Acute myocardial infarction (AMI) is one of the most serious manifestations of coronary artery disease. It is the disease with the third highest fatality rate in developed countries such as Europe and America (1). In recent years, with the continuous development of medical science and technology as well as the change in lifestyles, the mortality of AMI has been decreased. However, the mortality rate of myocardial infarctions with the total ischemic time dropping to a certain extent failed to decrease. Major cause of this situation is the ischemia reperfusion induced myocardial injury and necrosis (2,3). Ischemia reperfusion injury can induce increased oxygen free radicals and calcium overload in myocardial cells, as well as promoting myocardial cell apoptosis and necrosis, with the clinical manifestations of reperfusion arrhythmia, enlarged infarct size and deterioration of heart function (4). In order to further reduce the mortality and complications of myocardial infarction, researchers have shifted their focuses on the myocardial protection.

Related studies show that mice with myocardial hypertrophy and cardiac dysfunction were associated with miRNA-214 overexpression (5), and overexpression of miRNA-214 can inhibit the progression of cardiac hypertrophy by inhibiting the expression of *EZH2* in cardiac myocytes (6). miRNA-214 could affect the apoptosis of cardiomyocytes induced by chronic intermittent hypoxia through regulating its target genes *HIF-1* and *Fas*, which were involved in the death receptor signaling pathway (7). From these we can see that miRNA-214 may regulate the biological characteristics of necrosis and apoptosis of cardiomyocytes by regulating its target genes. There are few reports on the expression of miRNA-214 in the sera of elderly patients with AMI and the mechanism of its expression on cardiomyocyte necrosis and apoptosis. Therefore, we detected the expressions of sera miRNA-214 in the elderly AMI, UA and healthy elderly people and established human cardiac cell lines overexpressing miRNA-214, in order to study the expression of miRNA-214 in elderly patients with myocardial infarction and explore its effect on the apoptosis of myocardial cells.

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Materials and methods

Clinical materials. A total of 68 cases of elderly patients were randomly selected from the patients admitted in our hospital from February 2016 to October 2016, in which 43 cases were with acute myocardial infarction (AMI) and 25 cases were with unstable angina (UA). In addition, 25 cases of healthy elderly people who received physical examinations in our hospital during the same period of time were selected as the control group. The present study was approved by the Ethics Committee of Jinling Hospital Affiliated Nanjing University (Nanjing, China) and all patients provided written informed consent for study participation.

Inclusion criteria: i) aged over 65 years old; ii) AMI patients were diagnosed as AMI by WHO diagnostic criteria (8); iii) UA patients were diagnosed with UA according to the 'guidelines for the diagnosis and treatment of unstable angina pectoris and non ST segment elevation myocardial infarction' prepared by the cardiovascular branch of the Chinese Medical Association (9); iv) blood samples of AMI patients were obtained within 12 h of the onset of myocardial infarction related clinical symptoms; and v) the blood samples of UA patients were taken at the time of admission.

Exclusion criteria: i) the time of onset is unknown or AMI patients whose blood samples were not taken within 12 h of onset; ii) patients with combined diseases of malignant tumor, cerebrovascular disease or diseases of liver, kidney and other organs; iii) patients with unknown medical histories; iv) AMI and UA patients who did not die after treatment; and v) patients with the BMI less than 18.5 kg/m² or greater than 24 kg/m².

Blood Samples and plasma index. Peripheral blood (5-10 ml) was collected with EDTA-containing, then centrifuged at 1,000 x g for 10 min (5810R; Eppendorf, Hamburg, Germany) to collect plasma. And the plasma levels of AST and LDH were measured by an automatic biochemical analyzer (AU680; Beckman Coulter, Inc., Brea, CA, USA). The plasma levels of AST and LDH were measured by AST Assay kit (AmyJet Scientific, Inc., Wuhan, China) and LDH Assay kit (AmyJet Scientific, Inc.), respectively.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from 200 μ l plasma and 1,000 μ l QIAzol lysis reagent (Qiagen GmbH, Hilden, Germany), stand for 15 min in room temperature, and then extracted the RNA according to the protocol in miRNeasy sera/Plasma kit (Qiagen GmbH). Finally, 15 μ l DEPC water was used to solute the total RNA.

cDNA was synthesized from RNA which was isolated as before by One Step PrimeScript miRNA cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan). PCR parameter settings: 37°C for 60 min and 85°C for 5 sec.

RT-qPCR: 20 μ l RT-qPCR system was prepared with SYBR Premix Ex TaqTM II (Takara Bio, Inc.), and ABI 7500 Fluorescence Quantitative PCR Instrument (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to expand. PCR parameter settings: 95°C for 30 sec, 40 cycles of 90°C for 5 sec and 65°C for 30 sec. U6 was chosen as internal reference, and the relative expression of miR-214 was calculated by 2^{- $\Delta\Delta C_q$} method ($\Delta q = C_{q_{miRNA}} - C_{q_{U6}}$) (10).

HCM cell culture and treatments. Human cardiomyocytes (HCMs; cat. no. HZ-H358; Shanghai Huzhen Biotechnology Co., Ltd., Shanghai, China) were passaged one day before transfection (so that the cell density was 70-90% on the day of transfection). miRNA-214 mimic (5'-ACAGCAGGCACA GACAGGCAG-3', synthesized by Sangon Biotech Co., Ltd., Shanghai, China) and Negative control (5'-UUCUCCGAA CGUGUCACGUTT-3', synthesized by Sangon Biotech Co., Ltd.) miRNA was transfected using Lipofectamine 2000 liposomes. The medium was replaced with normal culture medium 6 h after transfection, and one group of the transfected cells was treated with H₂O₂ (final concentration 100 μ M) 24 h after transfection while the other group of cells was treated with the equal amount of sterile water. After cultured for another 24 h, cells were detached and collected.

Detection of human cardiomyocytes apoptosis. The Annexin V-FITC/PI kit (Invitrogen, USA) was used for flow cytometry to detect apoptosis. The experimental procedure refers to their protocol: The cells were centrifuged at 800 x g for 3 min at room temperature. The supernatant was discarded and washed with D-Hank's. Centrifuge at 800 x g for 3 min and repeat washing twice. Aspirate the supernatant and discard it. Add a pre-diluted binding buffer to the cell pellet at the bottom of the tube and mix well with 500 μ l/well. Add 10 μ l of FITC-labeled Annexin V and 5 μ l of PI and mixed, incubated at room temperature in the dark for 10 min, and then tested on the Beckman CytoFLEX Flow cytometry (Beckman Coulter, Inc.).

Western blot detection of protein expressions. Proteins were extracted by total cellular protein extraction kit (Beyotime Institute of Biotechnology, Haimen, China) and the protein concentration was detected by BCA protein quantification kit (Beyotime Institute of Biotechnology). 75 μ g of total protein was loaded into each lane and separated by 15% SDS-PAGE (90V, 0.5 h; 120 V, 1 h) and transferred (400 mA, 1.5 h) to polyvinylidene fluoride film (Amersham BioSciences, Buckinghamshire, UK), fixed with methanol for 1 min, washed three times (5 min each) with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.6) and blocked with blocking buffer (5% skim milk in TBST) for 1 h. PUMA (ab9643, 1:1,000; Abcam, Cambridge, UK), PTEN (ab31392, 1:500; Abcam), Bax (ab53154, 1:1,000; Abcam), caspase 7 (ab25900, 1:1,000; Abcam) and β -actin (ab8227, 1:3,000; Abcam) antibody was diluted by 5% skim milk was added and incubated at 4°C overnight, washed with TBST for 3 times (10 min each). Sheep anti-rabbit secondary antibody (ab205718, 1:2,000; Abcam) was added and incubated for 1 h at room temperature, and then ECL solution was added for detection. The expression of the target protein was analyzed by Image J software, and the relative expression level of target protein was characterized by the gray value of the target protein/gray value of β -actin protein.

Statistical analysis. Statistical analysis was performed using the SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). Student's t-test or one-way analysis of variance (ANOVA) was used to analyze the differences between groups. For one-way ANOVA, Duncan's post hoc test used following ANOVA to compare specific groups for multiple comparisons.

Table I. comparison of general data of the three groups of study subjects.

| Variable | AMI | UA | HE | P-values |
|--------------------------|-----------|-----------|-----------|----------|
| Total no. of cases (n) | 43 | 25 | 25 | - |
| Age (years) | 69.2±7.2 | 68.7±4.9 | 69.5±6.6 | 0.256 |
| Male (n/%) | 23/53.5 | 14/56.0 | 13/52.0 | 0.847 |
| BMI (kg/m ²) | 21.9±5.6 | 22.1±7.2 | 21.8±5.9 | 0.104 |
| Smoking (n/%) | 20/46.5 | 11/44.0 | 13/52.0 | 0.964 |
| Hypertension (n/%) | 34/79.1 | 19/76.0 | 20/80.0 | 0.113 |
| TC (mmol/l) | 4.09±0.72 | 3.91±1.18 | 3.88±0.74 | 0.089 |
| TG (mmol/l) | 1.72±0.54 | 1.59±1.21 | 1.44±0.76 | 0.094 |
| HDL-C (mmol/l) | 1.15±1.08 | 1.11±0.86 | 1.25±0.46 | 0.152 |
| LDL-C (mmol/l) | 2.71±0.79 | 2.42±1.02 | 2.35±0.98 | 0.259 |

Data are presented as the mean ± standard deviation. AMI, Acute myocardial infarction patients; UA, unstable angina patients; HE, healthy people; BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

Pearson's analysis was used to examine the correlation between two indicators. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparisons between the general data of AMI, UA and healthy study subjects. We compared the age, sex, BMI, smoking status, hypertension and blood lipid indexes of the elderly patients with AMI, elderly patients with UA and healthy elderly subjects, and confirmed that there was no significant difference between the three groups of study subjects in the general data such as age, gender and BMI ($P>0.05$; Table I).

Comparison of miR-214 expressions in different study subjects. Blood samples were obtained from different groups of subjects, sera were separated and the relative expressions of miR-214 in the sera were detected by quantitative real-time PCR. The results showed that the relative expressions of sera miR214 in elderly AMI patients, elderly UA patients and healthy elderly people were 15.79 ± 4.66 , 4.60 ± 2.51 , and 2.07 ± 0.99 , respectively. There were statistically significant differences between groups ($P<0.05$), as shown in Fig. 1.

Correlation between miR-214 expression and sera markers in elderly patients with myocardial infarction. The relationship of relative expression of miRNA-214 in the sera of elderly AMI patients with the sera AST, LDH, CK-MB or cTnI levels was examined by Pearson correlation analysis. The results showed that the relative expression level of miRNA-214 in elderly AMI patients was positively correlated with the sera AST ($r=0.361$, $P=0.0174$), LDH ($r=0.425$, $P=0.0045$), CK-MB ($r=0.835$, $P<0.001$) and cTnI ($r=0.770$, $P<0.001$), as shown in Fig. 2. And we also found that miR-24 expression has nothing to do with the serum markers (AST, LDH, CK-MB and cTnI) in healthy people. Although miR-24 expression has nothing to do with the serum markers (AST, LDH, CK-MB and cTnI) in angina patients, we believed that miR-214 also plays a role in promoting myocardial cell apoptosis in unstable angina. Just

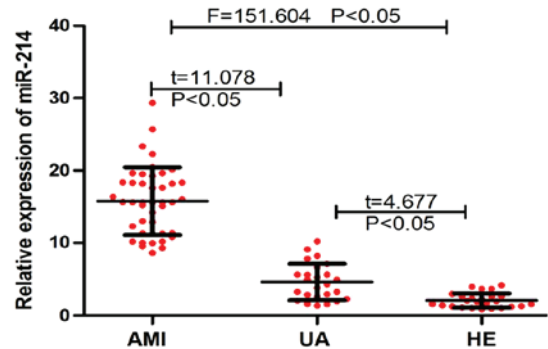


Figure 1. miR-214 expressions in different groups of subjects. $P<0.05$, as indicated. AMI, Acute myocardial infarction patients; UA, unstable angina patients; HE, healthy people; miR, microRNA.

because there was no abnormal or little changes in CKMB, troponin, and other cardiac enzymes of the unstable angina patients, there was no direct linear correlation between miR-214 expression and the serum markers in unstable angina patients.

The effect of miR-214 expression on HCM apoptosis. H_2O_2 is a strong oxidizing agent which can induce apoptosis if added into the cell culture medium. We found in this study that without H_2O_2 treatment, HCMs transfected with negative control or miR-214 mimic showed no significant difference in apoptosis rates ($P>0.05$), while after H_2O_2 treatment, the apoptosis rate of HCMs transfected with miR-214 mimic was significantly lower than that of negative control group ($P<0.05$), as shown in Fig. 3.

The effect of miR-214 expression on the expressions of apoptosis-related proteins in HCMs after H_2O_2 treatment. Western blot was performed to detect the effect of miR-214 expression on the expressions of PUMA, Bax, PTEN and caspase 7 proteins in HCMs after H_2O_2 treatment. The results showed that compared to negative control group, HCMs transfected with miR-214 mimic exhibited downregulated expressions of

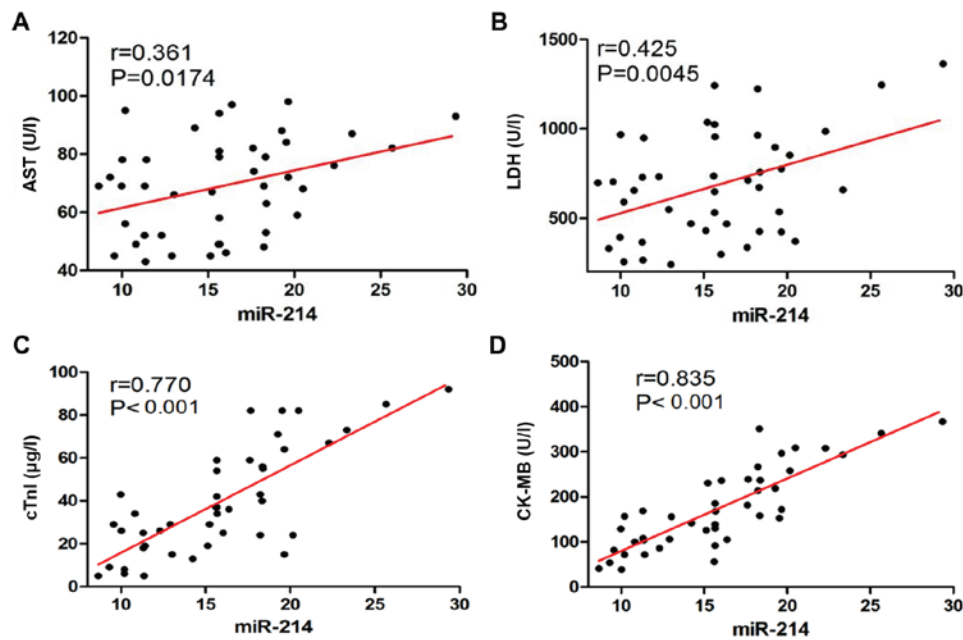


Figure 2. Correlation between miR-214 expression in elderly AMI patients and serological markers. Correlation between (A) miR-214 and AST ($r=0.361$, $P=0.0174$); (B) miR-214 and LDH ($r=0.425$, $P=0.0045$); (C) miR-214 and cTnI ($r=0.770$, $P<0.001$); and (D) miR-214 and CK-MB ($r=0.835$, $P<0.001$). AMI, Acute myocardial infarction patients; miR, microRNA; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CK-MB, creatine kinase-MB; cTnI, cardiac troponin.

PUMA, Bax, PTEN and caspase 7 proteins by 63.64, 21.95, 46.67 and 50.05%, respectively (Fig. 4).

Discussion

AMI is a clinically common myocardial necrosis disease caused by acute or persistent ischemia and hypoxia of the coronary arteries. As the disease is characterized by rapid onset and high mortality, it is one of major causes of death in patients. In recent years, with the development of AMI research, the pathogenesis of AMI has become clear. Most of myocardial infarction is caused by unstable atherosclerotic plaque rupture or erosion of coronary artery endothelium (11). After the rupture of an unstable atherosclerotic plaque, the release of the thrombus causing factor causes platelet activation, initiates the coagulation cascade, leads to mural thrombosis and leads to embolism under atherosclerotic plaques (5,6). This hypercoagulable state may contribute to rupture of other unstable fibrous cap plaques, resulting in myocardial cell necrosis, which can be detected by elevated biomarkers of peripheral blood. In recent years, many researchers have found that miRNA plays an important role in modulating myocardial function and cardiomyocytes apoptosis and necrosis through regulation of the expression of its target genes, and that it participates in various physiological processes in the pathogenesis of AMI and could be used as a potential peripheral blood biomarker in the diagnosis of AMI (12,13).

miRNA is a short (20-24 nt) non-coding RNA that regulates post transcriptional regulation of gene expressions in multicellular organisms by affecting the stability and translation of mRNA. It is an ideal blood biomarker for the detection of diseases because it is very stable in the blood and is often differentially expressed under various pathophysiological conditions. In this study, we detected the sera miRNA-214

expressions in different groups of subjects, and the results showed that the expression of miRNA-214 was significantly higher in the sera of elder AMI patients than that of elderly UA patients and healthy elderly people ($P<0.05$), which suggested that miRNA-214 could act as a potential serological markers for AMI diagnosis. In order to further investigate the value of miRNA-214 as a serological marker for AMI diagnosis, we analyzed the correlations of miRNA-214 with AST, LDH, CK-MB and cTnI, and the results showed that the expression of miRNA-214 in the sera of elder AMI patients was all positively related to AST, LDH, CK-MB and cTnI ($P<0.05$). AST, LDH and CK-MB belong to a group of enzymes that are closely related to myocardial injury, which are all increased to different extents upon myocardial injury or necrosis and therefore they are of certain diagnostic values for myocardial infarction. cTn protein isoforms I and T have become the preferred diagnostic biomarkers because they have high sensitivity and specificity on myocardial injury. Their up-regulations can be detected within 2-3 h, which reach the peak values within 24-28 h. Therefore, they are the preferred biomarkers for the diagnosis of acute myocardial infarction, as highlighted in the guidelines in Europe and the United States (14).

From the above studies, we can see that the expression of miRNA-214 is positively related to sera AST, LDH, CK-MB and cTnI levels, which suggests that miRNA-214 may be a miRNA associated with myocardial injury or necrosis. The study of Lv *et al* (15) in HCMs showed that miRNA-214 was highly expressed in the H_2O_2 induced cardiomyocyte apoptosis model, and that overexpression of miRNA-214 could inhibit cardiomyocyte apoptosis. In this study, the HCM cell line expressing miRNA-214 was established by transfection of miRNA-214 mimic. After H_2O_2 induction, we obtained the same result as that of Lv *et al* (15). The study by Aurora *et al* (16) revealed some of the mechanism of how

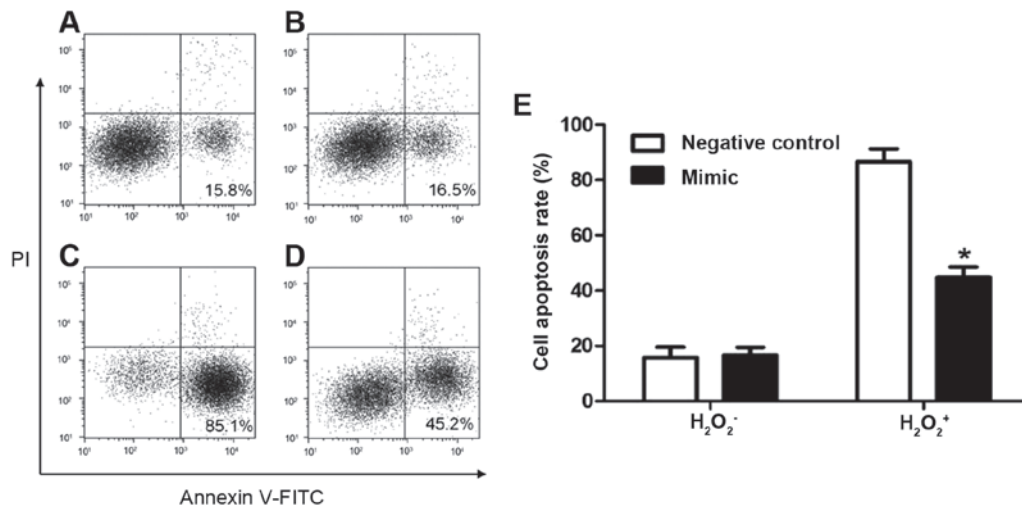


Figure 3. miR-214 reduces the apoptotic rate of HCMs following H₂O₂ treatment. The flow cytometry results of HCMs transfected with (A) negative control without H₂O₂; (B) with miR-214 mimic without H₂O₂; (C) with negative control and H₂O₂; and (D) with miR-214 mimic and H₂O₂. In flow cytometry, the lower right quadrant refers to apoptotic cells. (E) Apoptotic rate of HCMs. *P<0.05 vs. negative control. miR, microRNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; HCMs, human cardiomyocytes.

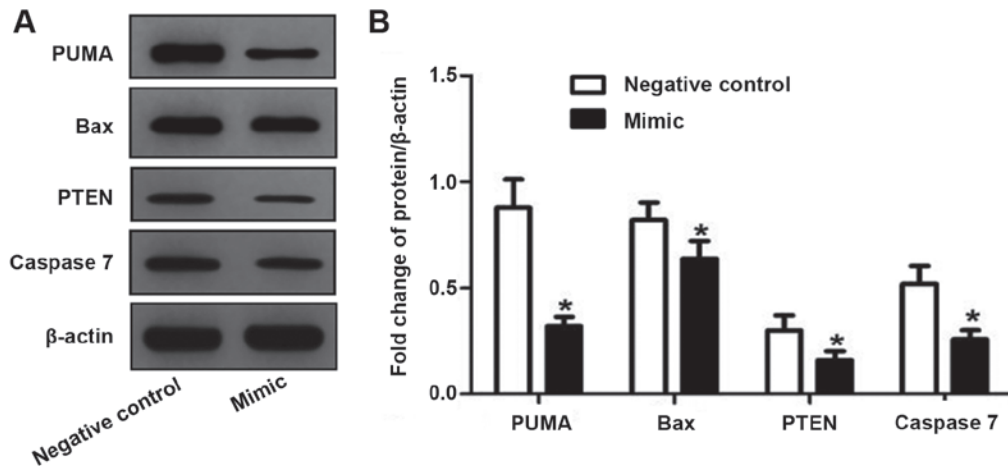


Figure 4. Effect of miR-214 expression on the expressions of apoptosis-associated proteins in human cardiomyocytes following H₂O₂ treatment. (A) Western blot assay of PUMA, Bax, PTEN, Caspase 7 and β-actin expression in the negative control and mimic groups. (B) Fold-change of protein/β-actin. *P<0.05 vs. the negative control. miR, microRNA; PUMA, p53-upregulated modulator of apoptosis; Bax, B-cell lymphoma-2-associated X protein; PTEN, phosphatase and tensin homolog.

miRNA-214 inhibits cardiomyocyte apoptosis. Their research showed that miRNA-214 was highly expressed in the blood of patients with ischemic injury and heart failure, which could inhibit the expression of sodium/calcium exchanger channel 1 related proteins and reduce the intracellular calcium imbalance caused by myocardial injury, thus resulting in the protection of myocardial cells. In combination with our results, it can be concluded that the high expression of miRNA-214 in the sera of elderly patients with AMI has a protective effect on cardiomyocytes.

We searched for possible targets of miRNA-214 through Targetscan, CoGeMiR, miRWalk and other miRNA target gene prediction sites or software, and selected 4 genes related to apoptosis of cardiomyocytes, which are PUMA, PTEN, Bax and caspase 7. Our results showed that after H₂O₂ treatment, the expressions of puma, PTEN, Bax and caspase 7 proteins in HCM cells overexpressing miRNA-214 was down-regulated

by 63.64, 21.95, 46.67 and 50.05%, respectively, compared with normal HCM cells. *Puma*, *PTEN*, *Bax* and *caspase 7* are all apoptosis-related genes. *Puma* is a member of the Bcl-2 family, which can bind with other members of the Bcl-2 family and induce changes of mitochondrial membrane potentials, thus leading to cell apoptosis. Moreover, Choy *et al* (17) have proved that *puma* is a target gene directly regulated by miRNA-214. The targeted regulation of *PTEN* by miRNA-214 has also been well demonstrated in the studies of various types of tumors (18,19), and the study of Lv *et al* (15) has shown that *PTEN* acts as a direct regulation target of miRNA-214 in human cardiomyocyte models. *Bax* and *caspase 7* are both classic apoptotic genes. *Bax* belongs to the Bcl-2 family while *caspase 7* belongs to the caspase protein family. Both of their molecular mechanisms in promoting apoptosis have been well understood, and relevant studies have confirmed that they are both direct targets for miRNA-214 regulation (20,21).

Taken together, our results demonstrated that miRNA-214 was highly expressed in the sera of elderly AMI patients, and that it could inhibit myocardial cell apoptosis by inhibiting the expressions of multiple target genes, such as *PUMA*, *PTEN*, *Bax* and *caspase 7*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY analyzed and interpreted the patient data. YY and WW performed the examinations. LL conceived and designed the study, and wrote and revised the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Jinling Hospital Affiliated Nanjing University (Jiangsu, China) and all patients provided written informed consent for study participation.

Patient consent for publication

All patients provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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