miRNA-21 Expression in the Serum of Elderly Patients with Acute Myocardial Infarction

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Background: The aims of this study were to examine the expression of miRNA-21 in the serum of elderly patients (>65 years) with acute myocardial infarction (AMI) and to investigate the potential role of serum miRNA-21 as a marker of early cardiac myocyte damage.

Material/Methods: Thirty-eight elderly patients with recent AMI, 27 elderly patients with unstable angina pectoris, and 25 healthy elderly individuals were included in the study. Serum miRNA-21 expression was determined following total RNA extraction and reverse-transcribed into cDNA, followed by reverse transcription-polymerase chain reaction (RT-PCR). Serum creatine kinase MB isoenzyme (CK-MB) and cardiac troponin I (cTnI) levels were analyzed by electrochemiluminescence. Apoptosis of human cardiac myocytes (HCM) was analyzed using fluorescence-activated cell sorting (FACS), and protein expression of caspase-3 was detected using Western blot.

Results: Expression levels of miRNA-21 in the serum of elderly patients with AMI were positively correlated with serum levels of CK-MB (r=0.3683, P=0.0229) and cTnI (r=0.5128, P=0.009). Following tumor necrosis factor (TNF)-α induction, the apoptosis rates of HCM transfected with the miRNA-21 mimic short hairpin RNA (shRNA) were downregulated by 39.1% compared with control HCM cells, and protein expression of c-Jun N-terminal kinases (JNK) and p38 were unchanged (P>0.05); protein expression of p-JNK, p-p38 and caspase-3 were downregulated by 37.1%, 35.8%, and 36.0%, respectively.

Conclusions: Expression of miRNA-21 was upregulated in the serum of elderly patients with AMI, which inhibited TNF-α induced apoptosis in HCM by activating the JNK/p38/caspase-3 signaling pathway.

MeSH Keywords: Apoptosis • Frail Elderly • MicroRNAs

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Background

Acute myocardial infarction (AMI) results in myocardial cell necrosis and is usually caused by occlusion of the coronary arteries, resulting in chronic morbidity or death. In developed countries, including Europe and the United States, the annual incidence of new patients with AMI is estimated to be 1,500,000 [1]. Recently, with the continued development of China's economy and the acceleration of urbanization and the increasingly aging population, the incidence of chronic disease, including ischemic heart disease, is increasing annually [1].

According to published data in the 2015 Report of Cardiovascular Diseases in China, released by National Cardiovascular Disease Center [2], by the end of 2015, the number of myocardial infarctions in China exceeded 2,500,000 while the morbidity and mortality rates of AMI showed an annually increasing trend. At present, the clinical diagnosis of AMI is still dependent on the clinical symptoms, changes in the electrocardiogram (ECG), and the increased levels of serum markers for myocardial necrosis. Recent studies of detection of AMI in recent years, a large number of sensitive and specific biomarkers for early diagnosis and prognosis of AMI have been found, among them is serum detection of miRNA-21 [3].

Studies have shown that miRNA-21 is a widely expressed miRNA regulated by RNA polymerase II and has been found in many tissues, including the heart, small intestine, and ovary [3]. Research studies in oncology have shown that miRNA-21 participates in the regulation of tumor cell proliferation, apoptosis, and migration, and may also be involved in carcinogenesis by inhibition of cell senescence [4–6]. Published studies on cardiovascular disease have shown that increased serum levels of miRNA-21 have been found in patients suffering from cardiovascular disease, including heart failure, aortic stenosis, and myocardial ischemia [7,8]. In an animal model of acute myocardial infarction (AMI), it has been shown that the expression of miRNA-21 was downregulated in the infarcted area (P<0.05) and that overexpression of miRNA-21 could protect myocardial cells from entering apoptosis by targeting apoptosis protein 4, heat shock protein (HSP), and nitric oxide synthase (NOS), thus reducing the size of the myocardial infarction [9]. A further study has shown that increased expression of miRNA-21 could suppress the activation of the PTEN/AKT/FOXO3a signaling pathway, and could TNF-α-induced apoptosis of neonatal rat cardiac myocytes [10].

The aims of this study were to examine the expression of miRNA-21 in the serum of elderly patients with AMI, patients with unstable angina (UA), and healthy elderly people, and to establish a human cardiac myocyte (HCM) cell line that overexpressed miRNA-21 to investigate its potential role as a marker of early cardiac myocyte damage.

Material and Methods

Study participants

The protocol for this study was approved by the local ethics committee of our hospital. All study participants provided written informed consent. Thirty-eight elderly patients, aged more than 65 years, who had an acute myocardial infarction (AMI), 27 elderly patients with unstable angina (UA), and 25 healthy elderly (HE) people were randomly selected. The blood of patients with AMI was obtained within 12 hours of the onset of symptoms, the blood of patients with UA was obtained immediately after hospital admission, and fasting morning blood samples were obtained from the HE study participants. For each study participant, levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured. Study participants were noted to be taking a range of medications, including nitroglycerin, metoprolol. amlodipine besylate, thiazide diuretics, and aspirin.

All study participants were more than 65 years old. Patients with AMI were included if they had been diagnosed with non-ST-elevation myocardial infarction (NSTEMI) according to the diagnostic criteria from the World Health Organization (WHO). Patients with UA had symptoms of angina without evidence of myocardial infarction. All healthy elderly (HE) participants were over 65 years old without a medical history of heart, liver, or kidney disease.

Patients were excluded from the study if they had a history of malignancy, liver, or kidney disease. Patients with AMI of unknown time of onset were excluded were excluded, as were individuals with an unknown clinical history.

Reagents and equipment used

The human cardiac myocyte (HCM) cell line was obtained from American Type Culture Collection (ATCC) (USA). The human creatine kinase-MB (CK-MB) detection kit was obtained from GENTAUR (CA, USA). The cardiac troponin I detection kit (cTnI) and the miRNeasy Mini Kit designed for purification of total RNA were obtained from Qiagen (USA). Reverse transcription-polymerase chain reaction (RT-PCR) kit was obtained from Takara (Japan). The antibodies, including p-p38 antibody (E-1), p38, p-JNK, and caspase-3 mouse monoclonal antibodies, goat anti-mouse secondary antibodies were obtained from Santa Cruz (USA). Applied Biosystems real-time quantitative PCR (Thermo Fisher, USA), flow cytometry MACSQuant® Analyzer 10 for flow cytometry (Miltenyi Biotec, Germany), and the Elecsys 2010 electrochemiluminescence analyzer (Roche, Switzerland) were used.

Table 1. Details of the elderly individuals included in this study, including the 38 cases of elderly patients with acute myocardial infarction (AMI), 27 cases with unstable angina (UA), and 25 healthy elderly (HE) individuals.

Observation index	AMI group	UA group	HE group	P
Number (cases)	38	27	25	–
Age (years)	68.3±7.2	69.1±5.4	68.9±6.6	0.139
Male (n/%)	19/50.0	13/48.1	13/52.0	0.721
Smoking (n/%)	12/31.6	8/29.6	8/32.0	0.964
Hypertension (n/%)	26/68.4	18/66.7	13/52.0	0.543
TC (mmol/L)	4.18±0.89	3.89±1.02	3.78±0.82	0.106
TG (mmol/L)	1.76±0.96	1.64±1.13	1.42±0.69	0.148
HDL-C (mmol/L)	1.12±1.23	1.11±0.86	1.23±0.54	0.264
LDL-C (mmol/L)	2.69±0.85	2.38±0.94	2.33±0.67	0.119

Extraction and detection of serum miRNA-21

Blood samples from the study participants were centrifuged at 1000 rpm for 10 minutes to obtain the serum. Total RNA in the serum was extracted using the total-RNA extraction kit and reverse-transcribed into cDNA, which then underwent reverse transcription-polymerase chain reaction (RT-PCR). The relative expression of miRNA-21 was calculated by $2-\Delta\Delta Ct$ ($\Delta\Delta Ct=(CTmiRNA-21-CTU6)$ for the AMI or UA group – $(CTmiRNA-21-CTU6)$ for the HE group.

Detection of serum CK-MB and cTnI

Serum samples from the study participants underwent CK-MB and cTnI detection using the Elecsys 2010 electrochemiluminescence analyzer. The methods used were according to the manufacturer's instruction manual.

Culture and treatment of human cardiac myocytes (HCM)

HCM cells were cultured in high glucose DMEM medium that included 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO_2. Cell transfection was performed 24 hours after the HCM cells were inoculated into 6-well plates at the concentration of 1×10^6 cells/well using Lip2000-mediated transient transfection of the miRNA-21 mimic short hairpin RNA (shRNA). Twenty-four hours after transfection of miRNA-21 mimic or negative control shRNA, the experimental group was treated with tumor necrosis factor (TNF)-α at a concentration of 10 µg/L, while the control group was treated with the same volume of sterile water for another 24 hours before cell collection for further experiments.

Flow cytometry analysis of apoptosis of HCM

The treated HCM cells were collected and fixed in 70% pre-cooled ethanol, prepared by using pre-cooled PBS and absolute ethanol, overnight at 4°C, After washing in PBS, the cells were stained with propidium iodide (PI), analyzed by flow cytometry and cell apoptosis was quantified.

Western blot analysis of protein expression

The treated HCM cells were collected and underwent protein extraction using a bicinchoninic acid assay (BCA) kit. Then, 70µg of protein in each sample underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a wet polyvinylidene difluoride (PVDF) membrane (fixed in methanol). The membrane was blocked with 5% non-fat milk powder in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h at room temperature and incubated with primary antibodies including mouse monoclonal antibodies to p38, p-JNK, β-actin, and caspase-3, at 4°C overnight. After incubation with secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse polyclonal antibody, the band density was analyzed by ImageJ software and normalized against β-actin levels.

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 statistical program, and cell counting was presented as percentages (mean ± standard deviation). The differences between two groups were compared via an independent t-test, and the differences between three groups were compared by one-way ANOVA. Pearson correlation analysis was used to study the relationship between miRNA-21 and CK-MB/cTnI. $P<0.05$ was considered statistically significant.
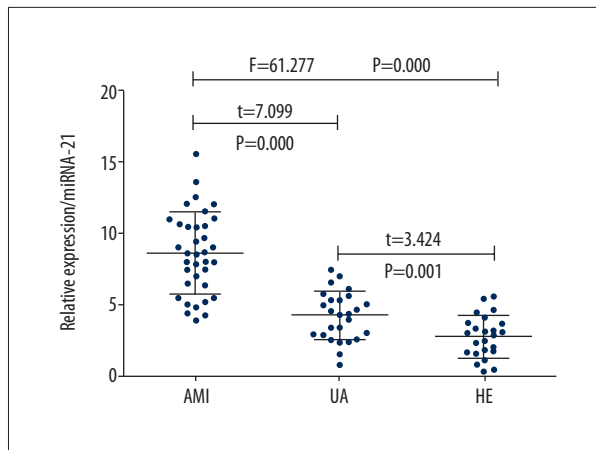
Figure 1. Relative expression of miRNA-21 in different study groups.

Results

Data on the elderly patients with acute myocardial infarction (AMI), unstable angina (AU) and healthy elderly (HE) individuals

The details of the elderly individuals included in this study, including the 38 cases of elderly patients with acute myocardial infarction (AMI), 27 cases with unstable angina (UA), and 25 healthy elderly (HE) individuals are shown in Table 1. There was no significant difference between the three groups in age, gender, smoking, blood pressure, or plasma levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), or low-density lipoprotein cholesterol (LDL-C) (P>0.05).

Relationship between miRNA-21 expression and CK-MB/cTnI content in the serum of elderly patients with AMI

As shown in Figure 1, the relative expression of miRNA-21 in the serum of elderly patients with UA patients was (4.3±1.7), which was significantly lower than that of elderly patients with AMI (8.6±2.8) and significantly greater than that of healthy elderly people (2.8±1.4). Pearson correlation analysis of the relative expression level of miRNA-21 and the CK-MB/cTnI content in the serum of elderly patients with AMI showed that there was a positive correlation between the relative expression level of miRNA-21 and CK-MB (r=0.3683, P=0.0229) or cTnI (r=0.5128, P=0.009) in the serum of elderly patients with AMI, as shown in Figures 2 and 3.

Effect of miRNA-21 expression on the rate of apoptosis in cultured human cardiac myocytes (HCM)

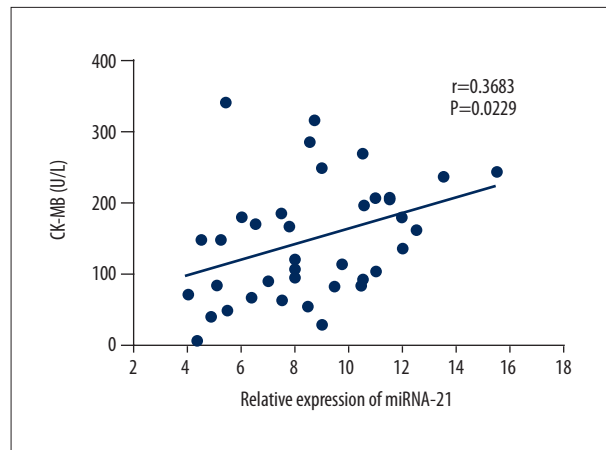The apoptosis rate of HCM transfected with miRNA-21 mimic, or negative control shRNA was (18.0%±5.4) (Figure 4B)

Figure 2. Correlation of the relative expression of miRNA-21 and CK-MB in the serum of elderly patients with acute myocardial infarction (AMI).
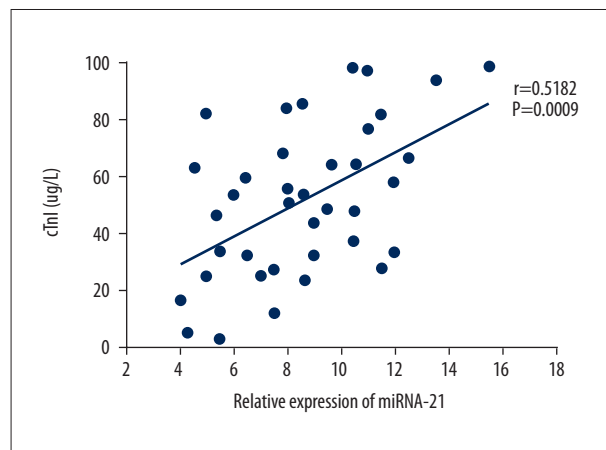

Figure 3. Correlation of the relative expression of miRNA-21 and cTnI in the serum of elderly patients with acute myocardial infarction (AMI).

and (18.8%±4.2) (Figure 4C), respectively. There was no significant difference (P>0.05) between the two groups, but the apoptosis rate was significantly greater than that of normal HCM cells (7.16%±2.5) (Figure 4A–4C) (P<0.05). After 24h of TNF-α treatment, the apoptosis rate of normal HCM cells was increased (79.2%±10.2) (Figure 4D), which was significantly greater than that of the mimic group (48.2%±10.7) (Figure 4E) (P<0.05) and significantly lower than that of the negative control group (87.3%±911.6) (Figure 4F) (P<0.05).

Effect of miRNA-21 expression on the mitogen-activated protein kinase (MAPK) signaling pathway proteins and caspase-3

Western blots were performed to detect the expression levels of mitogen-activated protein kinase (MAPK) signaling pathway proteins and caspase-3 protein in the three study groups. The
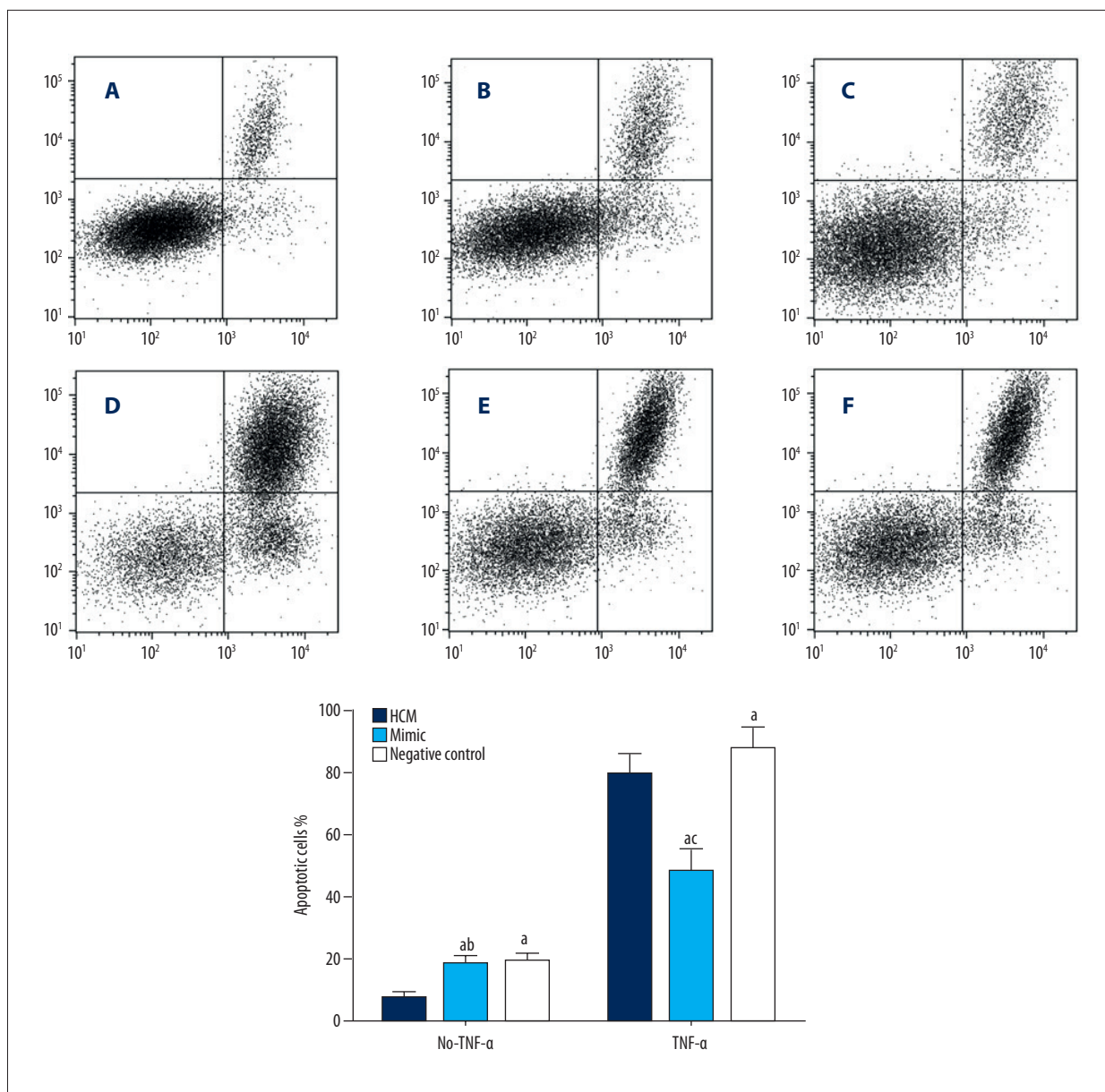
Figure 4. Fluorescence-activated cell sorting (FACS) analysis of the apoptosis rate of different groups of human cardiac myocytes (HCM). **A–C** represents the apoptosis rate of the HCM group, mimic group, and negative control group without TNF-α treatment, respectively. **D–F** shows the apoptosis rate of the TNF-α treated HCM group, mimic group and the negative control group, respectively. (1) Human cardiac myocyte (HCM) group: HCM cells without any treatment; (2) Mimic group: HCM cells transfected with miRNA-21 mimic shRNA; (3) Negative control group: HCM cells transfected with negative control shRNA; (4) [a] Shows that there was a statistically significant difference compared with HCM group (P<0.05); (5) [b] Shows that there was no significant difference compared with the negative control group (P>0.05); (6) [c] Shows that there was a significant difference compared with the mimic group (P<0.05).

results showed that, compared with the normal HCM group, the mimic group with miRNA-21 overexpression exhibited unchanged Jun N-terminal kinase (JNK) and p38 protein expression (P>0.05), while the expression of p-JNK, p-p38 and caspase-3 proteins were downregulated by 37.1%, 35.8%, and 36.0%, respectively. The expression levels of JNK, p38, p-JNK,

p-p38 and caspase-3 proteins were not significantly different from those in the normal HCM group (P>0.05) (Figure 5).
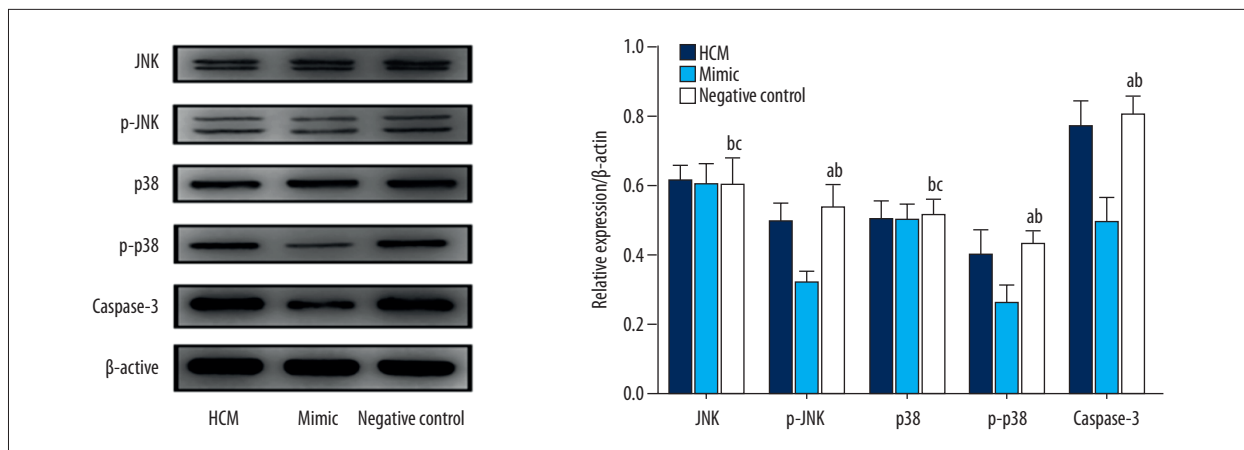
Figure 5. Effect of miRNA-21 expression on the expression of MAPK signaling pathway proteins and caspase-3 protein. (1) HCM group: HCM cells without any treatment; (2) Mimic group: HCM cells transfected with miRNA-21 mimic shRNA; (3) Negative control group: HCM cells transfected with negative control shRNA; (4) [a] Shows that there was a statistically significant difference compared with mimic group (P<0.05). (5) [b] Shows that there was no significant difference compared with HCM group (P>0.05). (6) [c] Shows that there was no significant difference compared with mimic group (P>0.05).

Discussion

In this study, we found that the expression of miRNA-21 in the serum of elderly patients with acute myocardial infarction (AMI) was significantly greater than in elderly patients with unstable angina (UA), and healthy elderly (HE) people (P<0.05). This study also showed that the level of expression of serum miRNA-21 in elderly patients with AMI was positively correlated with serum CK-MB (r=0.3683, P=0.0229) and cTnI (r=0.5128, P=0.009). CK-MB is one of the isoforms of CK, derived from the myocardium and is an important clinical diagnostic indicator in patients with AMI [11]. Also, cTnI is one of the three most important subunits of cardiac troponin, which has been widely used in the diagnosis and differential diagnosis of myocardial infarction and angina pectoris due to its high specificity and sensitivity [11]. The findings of this study support the possibility that increased expression of miRNA-21 in the serum of elderly patients with AMI may be related to the apoptosis of myocardial cells, and could be a potential diagnostic marker for AMI.

The human miRNA-21 gene is located on chromosome 17q23.2 and is constantly and highly expressed in vascular smooth muscle cells, cardiac myocytes, and cardiac fibroblasts [7,8]. The aberrant expression of miRNA-21 is involved in the development of cardiovascular disease [7,8]. TNF-α promotes T-cell function in inflammation, and previous studies have shown that TNF-α was highly expressed in the progression of cardiovascular disease such as heart failure, ischemic myocardial injury, AMI, and acute angina pectoris [12–14].

Overexpression of TNF-α and TNF-α receptors on the cell membranes of vascular endothelial cells, myocardial cells, and cardiac fibroblasts have been shown to induce cell apoptosis and participate in pathophysiological processes of cardiovascular disease [15,16]. Cardiac myocyte apoptosis is an important pathophysiological process in AMI, and inhibition of cardiac myocyte apoptosis has been proposed via the signaling transduction pathway [17,18]. In this study, we found that the apoptosis rate of TNF-α treated human cardiac myocytes (HCM) transfected *in vitro* with the miRNA-21 mimic shRNA was downregulated by 39.1%, which suggested that overexpression of miRNA-21 had protective effects on TNF-α-induced human cardiac myocyte (HCM) apoptosis *in vitro* and that miRNA-21 could be an important factor in cardiac myocyte protection during ischemia.

In order to further investigate the protective mechanism of miRNA-21 on TNF-α induced HCM apoptosis, we performed Western blots to detect the protein expression of Jun N-terminal kinase (JNK), p38 and caspase-3 as well as their phosphorylated forms in HCM cells. The results showed that the HCM cells with overexpression of miRNA-21 showed unchanged expression of JNK and p38 proteins (P>0.05) compared with normal HCM cells, while the expression of p-JNK, p-p38 and caspase-3 proteins were significantly inhibited (P<0.05).

The mitogen-activated protein kinase (MAPK) signaling pathway is composed of a group of evolutionarily conserved serine and threonine protein kinases, which can be activated by a series of extracellular stimuli and mediate the signal transduction from the cell membrane to the nucleus [19]. JNK/SAPK and p38 MAPK are two important mammalian signaling pathways. MAPK participates in the regulation of physiological processes such as apoptosis, proliferation, carcinogenesis and migration of tumor cells [19]. Previous studies have shown that the JNK/

SAPK pathway and p38 MAPK pathway share similar activators, including TNF-α, heat shock proteins, hydrogen peroxide and ultraviolet light [19]. In this study, we found that overexpression of miRNA-21 did not affect the expression of JNK and p38 in myocardial cells, but significantly inhibited the phosphorylation of these proteins. Since the degree of phosphorylation of JNK and p38 represents their activities, we inferred that miRNA-21 overexpression could inhibit the activation of the JNK/SAPK pathway and p38 MAPK pathway.

Activated caspase-3 can induce the expression of multiple proteins involved in apoptosis [20,21]. In the mitochondrial pathway of apoptosis, cytochrome-C is released from the mitochondrial outer membrane by the stimulation of apoptosis-related signals, cytochrome-C then activates caspase-9 by binding to apaf-1, and activated caspase-9 further activates caspase-3, which in turn activates caspase-6/7/8 and finally leads to cell apoptosis [20]. Somatostatin receptor type 2 (SST2) is an IL-33 receptor that can activate NF-κB, JNK/SAPK, and p38 MAPK pathways by binding to IL-33 in the blood to regulate cytokines including IL-2 and TNF-alpha to mediate inflammatory responses [22]. SST2 binds to IL-33 to mediate the inflammatory response and also to induce the rupture of the atherosclerotic plaque through the ST2/IL-33 pathway [23]. In this study, the expression level of miR-21 was also related to the activation of JNK/SAPK, p38 MAPK signaling pathways, and it is possible that it could also mediate inflammation. Although we may hypothesize that the expression of miR-21 in the blood may be similar to SST2 as a marker for the diagnosis of AMI and other cardiovascular disease, this hypothesis requires further investigation. We acknowledge that this was a small preliminary study with a small study population size using a single cardiac myocyte cell line. Further larger studies are required to evaluate the role of miRNA-21 as a potential serum marker for AMI.

Conclusions

The findings of this study showed that the expression of miRNA-21 was upregulated in the serum of elderly patients with AMI, which inhibited TNF-α induced apoptosis in HCM by activating the JNK/p38/caspase-3 signaling pathway that further inhibited TNF-α-induced apoptosis in cardiac myocytes cultured *in vitro*.

Conflict of interest

None.

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