Effect of miRNA-146 expression on inflammatory responses in elderly patients with acute myocardial infarction and in vitro

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Abstract: The study objective was to detect the expression of miRNA-146 in the serum of elderly patients with myocardial infarction and investigate the effect of its expression on the inflammatory response of peripheral blood in elderly patients with AMI and in vitro. Fluorescent quantitative PCR was performed to detect miRNA-146 expressions in the serums of 39 cases of elderly AMI patients, 21 cases of elderly Unstable angina (UA) patients and 21 cases of healthy elderly people (HE) that were admitted in our hospital from May 2016 to November 2016. The relationships between serum miRNA-146 expression and inflammatory factors such as serum CRP and TNF-α were analyzed. PBMC was isolated from peripheral blood of elderly patients with AMI, and the expression of miRNA-146 was up-regulated by transfection of miRNA-146 mimic. Western blot was performed to detect the effect of miRNA-146 overexpression on the expressions of SIRT1 and P65 proteins. The relative expressions of serum miRNA-146 in elderly AMI patients, elderly UA patients and healthy elderly people were (1.14±0.20), (0.32±0.056) and (0.19±0.039), respectively, and the differences between groups were statistically significant (P<0.05). The relative expression of serum miRNA-21 in elderly AMI patients was negatively correlated with serum CRP (r = -0.637, P<0.001) and TNF-α (r = -0.626, P<0.001). Transfection of miRNA-146 mimic up-regulated the expression of miRNA-146 in PBMC, which increased the expression of SIRT1 protein by 2.65 times while decreased the expression of nuclear p65 protein by 47.92% and the exocrine TNF-α by 57.77%. miRNA-146 was highly expressed in the serum of elderly AMI patients, which inhibited the inflammatory response of peripheral blood mononuclear cells in elderly AMI patients by inhibiting the NF-kB signaling pathway, thereby protecting the cardiomyocytes.

Keywords: miRNA-146, acute myocardial infarction, elderly, inflammatory response

Introduction

Acute myocardial infarction (AMI) is a myocardial necrosis caused by acute and persistent ischemia and hypoxia of coronary arteries. It is with high incidence in elderly people, and has the characteristics of high fatality rate and disability rate. Researches have shown that when myocardial infarction occurs, a large number of cardiomyocytes or other heart cells enter apoptosis and necrosis due to lack of oxygen and energy, and the cell contents released by lysis of necrosis cells can stimulate the immune system and induce severe inflammatory reactions [1]. The persistent inflammation not only leads to the degradation of extracellular matrix, but also continuously induces apoptosis of cardiomyocytes, which leads to necrosis and further enhances the inflammatory reactions. The vicious circle of inflammation and apoptosis can cause death of the patients if not treatment in time [2]. Therefore, in patients with acute myocardial infarction, timely and effective inhibition of the inflammatory response in vivo can reduce the apoptosis of cells thus reducing the damage cause by myocardial infarctions [3].

miRNA-146 is composed of two members, miRNA-146a and miRNA-146b, and is among the most widely studied micro RNAs. Studies [4] have shown that miRNA-146 is a gene with multiple functions, which play important roles in the physiological or pathological processes
of hematopoiesis, tumorigenesis, immunity and inflammation. Especially in the inflammatory response process, many studies showed that miRNA-146 was not only highly expressed in the tissues of osteoarthritis [5], prostate cancer [6] and inflammatory arthritis [7], but also its high expression can inhibit the inflammatory reaction through NF-kB signaling pathway [6, 8-10]. However, there are few reports about the expression of miRNA-146 in patients with acute myocardial infarction as well as the relationship between its expression and inflammatory response. Therefore, we detected the expression of serum miRNA-146 in patients with acute myocardial infarction as well as the relationship between its expression and inflammatory response. From these results, we further analyzed the relationship between serum inflammatory factors and the expression of miRNA-146 in elderly patients with AMI.

Materials and methods

Clinical materials

39 cases of elderly patients with myocardial infarction were randomly selected from the patients admitted in our hospital form May 2016 to November 2016. All the AMI patients were examined for electrocardiogram (ECG) and serum myocardial enzymes, which met the AMI diagnostic criteria established by WHO. During the same period of time, 21 cases of elderly unstable angina pectoris (UA) patients diagnosed in our hospital according to “unstable angina and non ST elevation myocardial infarction diagnosis and treatment guidelines” [9] and 20 healthy elderly people who received physical examinations in our hospital were also randomly selected as study subjects. In this study, we excluded the patients who were under the age of 60 years, and those who with liver or kidney dysfunction, chronic diseases, neurological movement disorders, severe arrhythmia, and cardiogenic shock, as well as those who died before receiving any treatment or whose blood was not collected within 12 hours of the onset of the disease. In addition, There was no significant difference between the three groups in terms of age, sex, body mass index, smoking status, combined hypertension, blood lipid or blood cholesterol (P<0.05), as shown in Table 1. This study was approved by the ethics committee of the First Affiliated Hospital of Soochow University and all patients provided written informed consent for study participation.

Reagents and instruments

Typsin, FBS, DMEM cell culture medium and 1640 cell culture medium (Hyclone, USA); miRNAeasy RNA isolation kit (Qiagen, USA); nuclear protein extraction kit (Beyotime Biotechnology, China); p65, SIRT1 and GAPDH rabbit polyclonal antibodies and HRP-labeled goat anti-rabbit polyclonal antibody (SANTACRUZ, USA); QuantStudioTM real-time quantitative fluorescence PCR system (ThermoFisher SCIENTIFIC, USA); flow cytometry-MACSQuant® Analyzer 10 (Miltenyi Biotec, Germany); SMT-100 portable automatic biochemical analyzer (Smarter, China).

PCR detection of the relative expression of miRNA-146

Blood samples were taken from elderly AMI or UA patients within 12 hours after the onset of the disease. After blood extraction, the serum and peripheral blood mononuclear cells should be separated and examined within 1 hour. The total RNA in the serum or peripheral blood mononuclear cells was extracted using miRNAeasy RNA isolation kit, and then reverse-transcribed into cDNA by reverse-transcription kit. The expression of miRNA-146 was detected by RT-PCR, and the relative expression level of miRNA-146 was calculated by 2^{-ΔΔCT}.

Determination of serological indexes

Blood samples were obtained from all the subjects and the serum was separated by centrifugation. Automatic serum biochemical analyzer...
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was used to detect the total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) as well as CK-MB and cTnI contents in the serum. The serum levels of inflammatory factors such as CRP and TNF-α were detected by the commercial kit (Shanghai Kang Lang biology, China).

Cell culture and treatment

HCMs (ATCC, USA) were culture using high glucose DMEM medium (+10% FBS), and passed one day before experiments. 24 hours after the passage, the HCMs were treated with different dosages of TNF-α (the final concentrations were 0 ug/L, 5 ug/L, 10 ug/L and 15 ug/L, respectively) for 24 h, and the cells were collected for the following experiments.

PBMCs of elderly AMI patients were separated by density gradient centrifugation, and inoculated at the density of 0.5×10^6/ml into 6-well plates (cultured with 1640 medium +10% FBS). Lipo 2000 was used to transfect miRNA-146 mimic/Negative control shRNA (Shanghai Bio-engineering Co., Ltd.) in to PBMCs. 24 hours after transfection, LPS (1 ug/ml) was added into the culture medium for 1 hour to stimulate the cells, and then the cells were collected and divided into 3 average parts. One part of the cells were subjected for total RNA extraction to detect miRNA-146 expressions, another part of the cells was subjected for total protein extraction and determination of SIRT1/GAPDH expressions by Western blot, and the third part was subjected for nuclear protein extraction and determination of p65 expression, while the supernatant of the cells was also collected for determination of miRNA-146, CRP and TNF-α contents.

Flow cytometry detection of HCM apoptosis

HCMs with different treatments were collected and fixed by pre-cooled 70% ethanol (prepared with pre-cooled PBS and absolute ethanol) at 4℃ over night. Then the cells were washed by PBS and stained with PI before the apoptosis rates were detected by flow cytometry.

Statistical analysis

Statistical analysis was performed using the SPSS19.0 statistical program. Measurement data was presented in the form of (mean ± standard deviation). Counting data was presented in the form of percentages. The differences between three groups were compared by single factor ANOVA, and t test was used to analyze the differences between groups. P<0.05 was considered statistically significant.

Results

No significant difference in the general information of three groups of subjects

The general data of 39 cases of elderly AMI patients, 21 cases of elderly UA patients and 20 cases of HE subjects was shown in Table 1. There was no significant difference between the three groups of study subjects in the general data such as age, gender, smoking status, combined hypertension, TC, TG, HDL-C or LDL-C (P>0.05).

miRNA-146 was highly expressed in the serum of elderly AMI patients

The blood of different groups of study subjects was obtained and the serum was separated. The relative expression of serum miR-146 was detected by real-time fluorescent quantitative PCR. The results showed that the relative expressions of serum miR-146 in elderly AMI patients, elderly UA patients and healthy elderly people were (1.14±0.20), (0.32±0.056) and (0.19±0.039), respectively, and the differences between groups were statistically significant (P<0.05), as shown in Figure 1.

Negative correlation between serum miRNA-146 expression and contents of inflammatory factors in elderly AMI patients

The relationship of relative expression of miRNA-146 in the serum of elderly AMI patients...
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Figure 2. The correlation of serum miR-146 expression with serum CRP/TNF-α in elderly AMI patients. A: The correlation of CRP and miR-146 (r = -0.637, P<0.001); B: The correlation of TNF-α and miR-146 (r = -0.626, P<0.001).

Figure 3. miRNA-146 inhibits the inflammatory response of peripheral blood mononuclear cells via NF-κB signaling pathway. A: miR-146 expression statistics in different cells; B: Effect of miR-146 expression on the expressions of SIRT1 and p65 proteins in peripheral blood mononuclear cells; C: Effect of miR-146 expression on the expression of TNF-α in peripheral blood mononuclear cells; *represented statistically significant differences compared to Negative Control group, P<0.05.

miRNA-146 mimic was (4.37±0.82), which was significantly higher than that of Negative control group (1.17±0.46) (P<0.05), as shown in Figure 3A.

The relative expression of SIRT1 protein in the PBMCs transfected with miRNA-146 mimic was (0.69±0.15), which was significantly higher than that in Negative control group (0.26±0.09) (P<0.05), while the expression of nuclear p65 protein (0.75±0.11) was significantly lower than that in Negative control group (1.44±0.19) (P<0.05), as shown in Figure 3B.

The TNF-α content in the supernatant of PBMCs transfected with miRNA-146 mimic was (24.00±9.47) ng/L, which was significantly lower than that of Negative control group (56.83±12.61) ng/L (P<0.05), as shown in Figure 3C.

Cardiomyocyte apoptosis induced by TNF-α

Different amounts of TNF-α were added into normal culture mediums of HCMs, and the effect of TNF-α on HCM apoptosis was detected by flow cytometry. The results showed that the apoptosis rates of HCMs without TNF-α treatment, or treated with 5 μg/L, 10 μg/L or 15 μg/L TNF-α were (5.3±0.8)%, (48.2±12.8)%, (78.4±10.2)%, and (92.1±7.6)%, respectively, as shown in Figure 4.

Discussions

miRNA-146 is the most widely studied miRNA and is also the first micro RNA found to have immunomodulatory effects. There are two members in the family: miRNA-146a, located on the human fifth chromosome (5q33), and miRNA-146b, located on the human tenth chromosome (10q24). The only difference between miRNA-146a and miRNA-146b is 2 different
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nucleotides in the 3' end [11]. The relationship between miRNA-146 and the chronic inflammatory diseases, atherosclerosis, and pointed out that miRNA-146 is closely related to the activation of inflammatory pathways in atherosclerosis [12]. At the same time, a number of studies also showed that miRNA-146 is highly expressed in multiple chronic inflammatory diseases, such as periodontitis [13], pulpitis [14], osteoarthritis [5] and psoriasis [15]. However, the expression of miRNA-146 in the serum of elderly patients with myocardial infarction is rarely reported both at home and abroad. In this study, we found that the relative expressions of serum miR-146 in elderly AMI patients, elderly UA patients and healthy elderly people were (1.14±0.20), (0.32±0.056) and (0.19±0.039), respectively, and there are statistically significant differences between the groups (P<0.05). These results indicated that miRNA-146 is highly expressed in the serum of elderly patients with acute myocardial infarction.

miRNA-146 is a small RNA that has a variety of functions, and many related researches have shown that it is highly expressed in many chronic inflammatory diseases [5, 13, 14], which plays a role in the inhibition of inflammatory reactions [6, 8-10]. Inflammatory reactions are closely related to the occurrence and development of acute myocardial infarction [1, 2]. On the one hand, the release of inflammatory mediators initiates the repair of the damaged tissues; on the other hand, inflammation reactions continuously induce matrix degradation and apoptosis of cardiomyocytes. Therefore, the high expression of miRNA-146 in the serum of elderly patients with AMI may be an intrinsic self-protection mechanism of the body-the inhibition of inflammatory responses by the high expression of miRNA-146. The following analysis in this study showed that the serum miRNA-146 in elderly patients with AMI was negatively correlated with the contents of inflammatory mediators, such as CRP and TNF-α, which supports the above conjecture.

TNF-α, a tumor necrosis factor, is the cytokine with the strongest anti-tumor activity ever found, and it is also an acute phase reactive protein that can promote T cells to produce a variety of inflammatory cytokines, thus promoting inflammatory reactions. Studies have shown that TNF-α is highly expressed in the progression of cardiovascular diseases such as

Figure 4. TNF-α induced apoptosis of cardiomyocytes was dose-dependent. The apoptosis rates of HCMs without TNF-α treatment, or treated with 5 ug/L, 10 ug/L or 15 ug/L TNF-α were (5.3±0.8)%, (48.2±12.8)%, (78.4±10.2)% and (92.1±7.6)%, respectively. A: The apoptosis rates of HCMs without TNF-α treatment were (5.3±0.8)%; B: The apoptosis rates of HCMs treated with 5 ug/L were (48.2±12.8)%; C: The apoptosis rates of HCMs treated with 10 ug/L were (78.4±10.2)%; D: The apoptosis rates of HCMs treated with 15 ug/L TNF-α were (92.1±7.6)%; E: The statistical analysis of apoptotic cells with different treatments.
heart failure, ischemic myocardial injury, acute myocardial infarction and acute angina pectoris [16]. In this study, we found that the addition of TNF-α into the cardiomyocytes culture medium could induce dose-dependent apoptosis of cardiomyocytes. The studies of Schenk B [17] and Wagner S A [18] have pointed out that the highly expressed TNF-α in the serum can bind with the TNF-α receptors on the cell membranes of multiple kinds of vascular cells such as cardiomyocytes, vascular endothelial cells and cardiac fibroblasts and induce cell apoptosis, thus participating in the pathophysiology of cardiovascular diseases. Therefore, we can conclude that high expression of miRNA-146 may protect cardiomyocytes by inhibiting the expression of inflammatory mediators in the serum.

To further investigate the mechanism of how miRNA-146 inhibits the inflammatory response in elderly patients with AMI, we isolated PBMCs from elderly AMI patients and transfected them with miRNA-146 mimic to promote the expression of miRNA-146. The results showed that overexpression of miRNA-146 not only increased the expression of SIRT1 protein and decreased the expression of nucleus p65 protein in PBMCs, but also inhibited the synthesis and secretion of TNF-α. SIRT1 (silent information regulator 1) is a histone deacetylase that is widely expressed in human cells, it carries out important biological functions by deacetylating multiple transcription factors, such as p53 [19], UCP2 [20], P300 [21] and NF-kB [18]. p65 is an important composition of NF-kB, which only functions after it is acetylated. In inflammatory responses, SIRT1 deacetylates p65 thus inhibiting the transcription of TNF-α, IL-6 and other inflammatory genes downstream of NF-kB [18]. p65 is an important protein in the TLR/NF-kB signaling pathway, and its phosphorylation-mediated translocation (from cytoplasm to nucleus) is an important marker of the activation of NF-kB signaling. In this study, we found that in the PBMCs overexpressing miRNA-146 (by transfection of miRNA-146 mimic), the expression of SIRT1 protein was significantly increased while the expression of nucleus p65 protein was significantly decreased. The study of Xie Y F et al. [22] indicates that LPS can promote the expression of miRNA-146 in human gingival fibroblasts, which inhibits the activation of NF-kB signaling pathway by inhibiting p65 phosphorylation, thus resulting in the down-regulation of IL-1, IL-6, TNF-α and other pro-inflammatory factors. Meanwhile, in the study of a mouse model of heart failure [9], it was shown that miRNA-146 was highly expressed in the serum of heart failure mice, and that overexpression of miRNA-146 could inhibit the NF-kB-dependent expression of ErbB4, thus inhibiting cardiomyocyte apoptosis by repressing the phosphorylation of Akt and Erk, downstream targets of ErbB4. From these results we can conclude that miRNA-146 may suppress the activation of the NF-kB signaling pathway in mammalian cells by inhibiting the phosphorylation of NF-kB pathway related proteins, thus resulting in the down-regulated expressions of multiple downstream proteins including pro-inflammatory cytokines.

In summary, MiRNA-146 was highly expressed in the serum of elderly AMI patients, which inhibited the inflammatory response of peripheral blood mononuclear cells in elderly AMI patients by inhibiting the NF-kB signaling pathway, thereby protecting cardiomyocytes.

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Disclosure of conflict of interest

None.

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