



Association between the level of CD4⁺ T lymphocyte microRNA-155 and coronary artery disease in patients with unstable angina pectoris

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Abstract

Objective To study the association between the expression of microRNA-155 (miRNA-155) in peripheral blood CD4⁺ T lymphocytes and the level of serum interferon- γ (IFN- γ) concentration and the severity of coronary artery disease (CAD). **Methods** After coronary angiography, 252 patients with suspected unstable angina pectoris (UAP) were divided into the UAP group (128 patients with CAD confirmed by angiography) and the control group (124 patients without CAD confirmed by angiography). Fresh peripheral blood was extracted 16–24 h before coronary angiography, CD4⁺ T lymphocytes was tested using immunomagnetic beads, the expression of miRNA-155 was tested using quantitative PCR and the expression of IFN- γ was tested using enzyme-linked immunosorbent assay (ELISA). According to the results of angiography, Gensini score of coronary artery lesions was analyzed. Furthermore, we also analysis the association between the level of miRNA-155 in peripheral blood CD4⁺ T lymphocytes, the level of serum IFN- γ and Gensini score of coronary lesion. **Results** The levels of miRNA-155 (0.49 ± 0.08 vs. 0.23 ± 0.09) and IFN- γ (227.58 ± 26.01 vs. 141.23 ± 17.89) in the UAP group were significantly higher than that of the control group, the difference was statistically significant. The level of miRNA-155 and IFN- γ were positively correlated with Gensini score of CAD ($r = 0.534$, $r = 0.713$, respectively, all $P < 0.05$). The level of miRNA-155 was positively correlated with the level of IFN- γ ($r = 0.686$, $P < 0.05$). **Conclusions** The level of miRNA-155 in peripheral blood CD4⁺ T lymphocytes and the level of IFN- γ are closely correlated with the severity of CAD.

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Keywords: CD4⁺ T lymphocyte; Coronary artery disease; IFN- γ ; MicroRNA-155

1 Introduction

Coronary atherosclerosis (CAS)^[1–3] is the main pathological basis of coronary heart disease. It can interrupt coronary blood flow, leading to symptoms and signs of myocardial ischaemia or infarction. Studies have shown that CD4⁺ T lymphocyte mediated immune response is involved in the occurrence and development of CAS.^[4–6] CD4⁺ T lymphocytes, especially the Th1 cells secreting interferon- γ (IFN- γ), can activate the macrophages in atherosclerotic plaques or directly affect the stability of plaque through the secretion of effector molecules, and then leading to acute coronary syndrome (ACS).^[7–9]

Micro-ribonucleic acid (miRNA) is deeply important in regulating gene expression. It is an endogenous, non-coding single stranded small-molecule RNA, which can be found in

eukaryotic organisms.^[10–12] Results have indicated that the length of miRNA is about 18–25 nucleotides, which are widely involved in the regulation of mammalian genes and play an important role in cell differentiation, proliferation, apoptosis and other biological processes.^[13] In recent years, it has been found that miRNA is involved in the regulation of T lymphocyte differentiation, proliferation and activation, the formation and development of CAS plaques.^[14]

In our previous studies,^[15,16] we found that the expression of CD4⁺ T lymphocyte microRNA-155 in peripheral blood of patients with unstable angina pectoris (UAP) was markedly increased, and it was involved in the occurrence and development of ACS by affecting the differentiation and function of Th1 cell. At present, there is little research on the association between miRNA-155 level and the severity of coronary artery disease (CAD). In addition, the mechanisms of miRNA-155 level and the severity of CAD is still unclear. Therefore, we investigated the association between the level of CD4⁺ T lymphocyte microRNA-155 in peripheral blood and the severity of coronary artery lesions in patients with UAP.

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2 Methods

Our research has obtained the approval of the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University, and all patients provided written informed consent. All patients were fully informed about the study protocol and signed informed consent before this study.

2.1 Study population

After coronary angiography, 252 patients with suspected unstable angina pectoris (UAP) treated in the First Affiliated Hospital of Guangxi Medical University from October 2011 to January 2016 were divided into the UAP group (128 patients with CAD confirmed by angiography) and the control group (124 patients without CAD confirmed by angiography). The diagnostic criteria for UAP are as follows: patients who were admitted with unstable coronary artery disease, which was defined as typical chest pain lasting > 10 minutes in the 24 h before admission, ECG changes, and/or raised serum levels of cardiac enzymes, including creatinine kinase and troponin. A non-Q-wave myocardial infarction (NQMI) was considered to be present on admission if troponin-I levels were 0.5 mg/mL within the first 12 h of hospitalization. At the same time, 124 patients with atypical angina pectoris and normal coronary angiography were enrolled as the control group. Inclusion criteria: (1) all patients are in accordance with the diagnosis of UAP, as we have described above, and undergoing percutaneous coronary intervention (PCI); (2) two groups of patients with myocardial enzyme markers negative (such as CK-MB and cTnI); (3) no contraindication of statin use; and (4) no history of contrast agent allergy. Exclusion criteria: (1) patient with severe infection or tumor; (2) patient with severe hepatic and renal dysfunction; (3) statin allergy; (4) cerebral apoplexy; (5) left ventricular ejection fraction < 30%; (6) patients undergoing emergency PCI; and (7) patient with heart valve disease.

2.2 Data collection

2.2.1 Specimen

20 mL peripheral venous blood samples for all participants were collected in EDTA-coated tubes 16–24 h before coronary angiography. Taking 1 mL venous blood of 20 mL fresh peripheral venous blood, after its natural coagulation for 20 min at normal atmospheric temperature, and then 2000rpm centrifugal for 10 min at 4°C to obtain platelet-poor plasma. Lastly, serum was collected for the detection of INF- γ using Enzyme-linked immunosorbent assay (ELISA). The remaining blood was used for the separation

of cells after heparin anticoagulation. All plasma samples were stored in aliquots at -80°C until RNA extraction.

2.2.2 Material

Human lymphocytoseparation (Solarbio, China), Dynabeads[®] FlowComp[™] Human CD4 kit (Dyna, Norway), RPMI1640 medium (Hyclone, USA), RNzol-A+ total RNA extraction reagent (Tian gen, China), RevertAid[™] First-Strand cDNA Synthesis kit (Fermentas, Lithuania), Fase Start SYBR Green Master (Rox, USA), MiRNA-155, U6 primer (GeneCopeia, USA), Human INF- γ Platinum ELISA (R&D, USA).

2.2.3 Cell extraction

The peripheral blood mononuclear cells (PBMC) of two groups were extracted according to Ficoll-Paque density gradient centrifugation, then resuspended in the 1 mL 1640 mediums. Taking 10 μL PBMC suspension cells, adding 90 μL PBS liquid diluted to 10 times and for a cell count. The remaining cells in strict accordance with the Dynabeads FlowComp it Human CD4 multisort kit instructions for sorting CD4⁺ T lymphocyte. Sorted cells also do cell count. At the same time, observe and calculate the survival rate of living cells after 0.4% trypan blue staining, the survival rate > 90% CD4⁺ T lymphocyte was reserved. PBMC of two groups were extracted according to Ficoll-Paque density gradient centrifugation, then resuspended in the mediums. Taking 10 μL PBMC suspension cells, adding 90 μL PBS liquid diluted to 10 times and for the cell count. The remaining cells in strict accordance with the Dynabeads FlowComp it Human CD4 multisort kit instructions for sorting CD4⁺ T lymphocyte. Sorted cells also do cell count. At the same time, observe and calculate the survival rate of living cells after 0.4% trypan blue staining, the survival rate > 90% CD4⁺ T lymphocyte was reserved.

2.2.4 Detection of miRNA-155 expression using fluorescence quantitative PCR

The total of RNA was extracted from CD4⁺ T lymphocyte according to the instructions of Trizol, and the concentration of RNA was measured by Nanodrop. At the same time, 1% agarose gel electrophoresis was used to detect the degradation of RNA. After that, the amount of RNA was adjusted to 1 $\mu\text{g}/\mu\text{L}$, reverse transcriptase synthesis cDNA. The PCR products were detected by SYBR Green induced fluorescence labeling method. The total reaction system was 20 μL , miRNA-155 and reference U6 primers were provided by GeneCopeia (primer ID were: hsmq-0290 and hsnRNAU6, respectively). The reaction system and pa-

rameters were set up according to the reagent kit, and all samples were detected by double holes. In addition, each reaction is set negative hole. The PCR product were all controlled by quality using sequencing, and the 2- $\Delta\Delta C_t$ method was used to calculate the relative expression level of microRNA-155 in all samples.

2.2.5 ELISA detection of INF- concentration

The serum samples, the culture medium supernatant and the ELISA kit were placed at room temperature about 30 minutes, and the operation procedure were carried out according to the instruction.

2.2.6 Method for evaluating the degree of coronary artery disease

The severity of coronary stenosis was assessed according to Gensini coronary artery score^[17,18]: stenosis $\leq 25\%$, 1 score; 25%–50%, 2 score; 50%–75%, 4 score; 75%–90%, 8 score; 90%–99%, 16 score; 100% (occlusion), 32 score. The score of coronary artery in different segments was scored according to Gensini standard. The final score of the degree of the coronary lesions in each patient was the sum of the scores of each branch. In addition, according to the results of coronary angiography, the patients were divided into mild, moderate and severe lesions. The specific method was as follows: 50%–70% stenosis was defined as mild lesions, 71%–90% lesions was defined as moderate lesions, 91%–100% stenosis was defined as severe lesions. According to the degree of lesions, the UAP group was divided into mild lesions ($n = 68$), moderate lesions ($n = 38$) and severe lesions ($n = 22$). The results of coronary angiography were evaluated independently by two experienced clinicians.

2.3 Statistical analyses

Data is presented as the mean \pm SD or number and percentage. Continuous variables were compared using either the Mann-Whitney U test or the Kruskal-Wallis test, whereas the one-way ANOVA and Tukey's test were used to compare more than two groups. For categorical variables, either the Chi-Square test or Fisher's test was used appropriately. Correlation analysis was used to investigate the correlations between the plasma levels of CD4⁺ T lymphocyte microRNA-155 and the severity of coronary artery disease. SPSS 23.0 software was used for all statistical analyses, and a two-tailed $P < 0.05$ was considered to be significant.

3 Results

3.1 General information of patient

There was no significant difference between the UAP

group and the control group in gender, age, cholesterol, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL), hypertension, diabetes, dyslipidemia, atrial fibrillation, smoking, stroke history, systolic blood pressure, diastolic blood pressure and history of drug use (all $P > 0.05$), as shown in Table 1.

3.2 MiRNA-155 level

Real time fluorescent quantitative PCR showed that the expression of miRNA-155 in mild (0.39 ± 0.12), moderate (0.54 ± 0.14) and severe lesions (0.68 ± 0.15) in the UAP group was significantly higher than that of the control group (0.23 ± 0.09), the difference was statistically significant ($P < 0.05$), as shown in Table 2.

3.3 Concentration of serum INF- γ

The results of ELISA showed that the serum INF- γ concentration in mild (184.21 ± 21.68 pg/mL), moderate (237.14 ± 27.96 pg/mL) and severe lesions (302.36 ± 32.12 pg/mL) in the UAP group was significantly higher than that of the control group (141.23 ± 17.89 pg/mL), the difference was statistically significant ($P < 0.05$), as shown in Table 3.

Table 1. Comparison of clinical characteristics between the two groups.

Variables	The Control group ($n = 124$)	The UAP group ($n = 128$)	P
Male	93 (75%)	100 (78%)	0.849
Age, yrs	63.87 \pm 10.13	65.18 \pm 10.97	0.837
Cholesterol, mmol/L	3.62 \pm 0.68	3.75 \pm 0.75	0.1511
Triglyceride, mmol/L	1.12 \pm 0.43	1.06 \pm 0.41	0.2579
HDL, mmol/L	1.32 \pm 0.58	1.28 \pm 0.55	0.5747
LDL, mmol/L	2.47 \pm 0.82	2.32 \pm 0.77	0.1355
Hypertension	52 (42%)	59 (46%)	0.733
Diabetes	26 (21%)	29 (23%)	0.882
Dyslipidemia	37 (30%)	45 (35%)	0.528
Atrial fibrillation	15 (12%)	15 (12%)	0.544
Smoking	46 (37%)	52 (41%)	0.722
Stroke history	14 (11%)	16 (12%)	0.848
Systolic blood pressure, mmHg	135.24 \pm 15.01	138.02 \pm 17.11	0.914
Diastolic blood pressure, mmHg	87.19 \pm 10.88	86.98 \pm 11.67	0.441
Treatment			
Aspirin	124	128	1.000
Clopidogrel	124	128	1.000
ACEIs/ARBs	93 (75%)	105 (82%)	0.704
Beta blockers	67 (54%)	74 (58%)	0.754
Calcium channel blocker	26 (21%)	31 (24%)	0.662
Nitrates	46 (37%)	55 (43%)	0.557

Data are presented as mean \pm SD or n (%). ACEIs: angiotensin-converting enzyme inhibitors; ARBs: angiotensin-receptor blockers; HDL: high density lipoprotein; LDL: low density lipoprotein.

Table 2. Comparison of the level of CD4⁺ T lymphocyte microRNA-155 in two groups.

Group	n	mir-155
The control group	124	0.23 ± 0.09
The UAP group	128	0.49 ± 0.08*
Mild lesions	68	0.39 ± 0.12
Moderate lesions	38	0.54 ± 0.14 [▲]
Severe lesions	22	0.68 ± 0.15 ^{▲, #}

Data are presented as mean ± SD. Compared with the control group, * $P < 0.05$; Compared with the mild lesions, [▲] $P < 0.05$; Compared with the moderate lesions, [#] $P < 0.05$. UAP: unstable angina pectoris.

Table 3. Comparison of the concentration of serum INF- γ in two groups.

Group	N	INF- γ , pg/mL
The control group	124	141.23 ± 17.89
The UAP group	128	227.58 ± 26.01*
Mild lesions	68	184.21 ± 21.68
Moderate lesions	38	237.14 ± 27.96 [▲]
Severe lesions	22	302.36 ± 32.12 ^{▲, #}

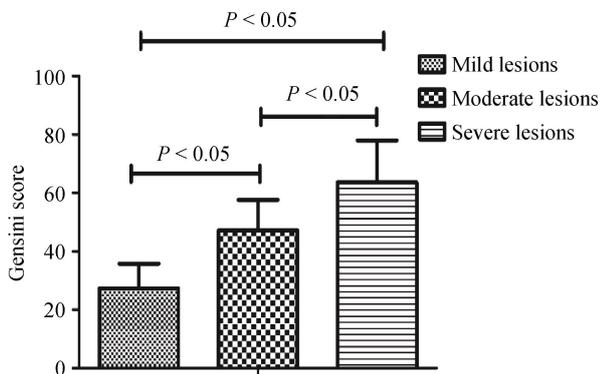
Data are presented as mean ± SD. Compared with the control group, * $P < 0.05$; Compared with the mild lesions, [▲] $P < 0.05$; Compared with the moderate lesions, [#] $P < 0.05$. UAP: unstable angina pectoris.

3.4 Gensini score of patients with different lesions in the UAP group

The Gensini score of patients with mild, moderate and severe lesions in the UAP group was 27.36 ± 8.41 , 47.13 ± 10.46 , 63.59 ± 14.37 , respectively. The difference between groups was statistically significant ($P < 0.05$), as shown in Figure 1.

3.5 Association between miRNA-155, INF- γ and severity of coronary artery disease in the UAP group

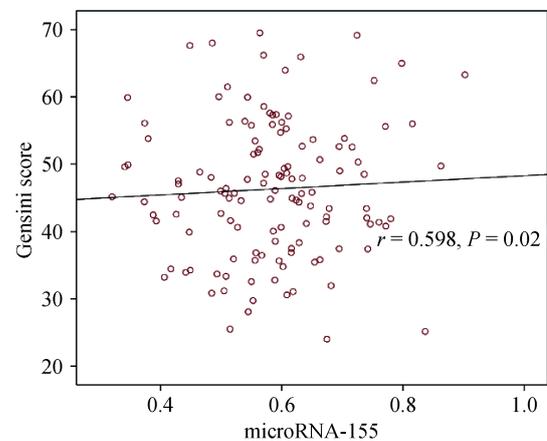
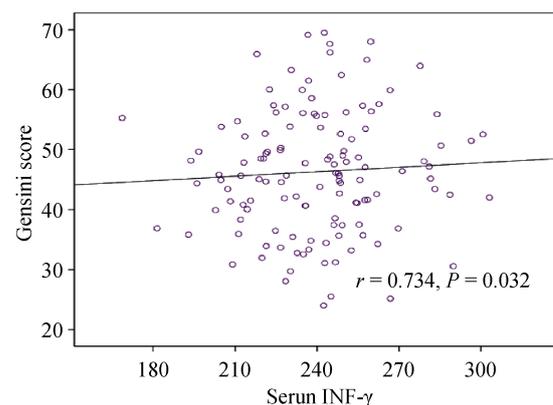
Correlation analysis showed that the level of CD4⁺ T

**Figure 1. Gensini score of patients with different lesions in the UAP group. UAP: unstable angina pectoris.**

lymphocyte microRNA-155 was positively correlated with Gensini score ($r = 0.598$, $P = 0.02$), as shown in Figure 2. Serum INF- γ level was positively correlated with Gensini score ($r = 0.734$, $P = 0.032$), as shown in Figure 3. In addition, there was a significant positive correlation between the level of CD4⁺ T lymphocyte microRNA-155 and the level of serum INF- γ ($r = 0.701$, $P = 0.000$), as shown in Figure 4.

4 Discussion

CAS is a kind of chronic inflammation or immune disease.^[19,20] The inflammatory cells and the secretion of inflammatory mediators and cytokines can cause plaque instability or rupture, thus leading to the occurrence of ACS. It was found that a large number of activated inflammatory cells were found at the sites where the plaques were prone

**Figure 2. Association between miRNA-155 and Gensini score in the UAP group. UAP: unstable angina pectoris.****Figure 3. Association between serum INF- γ level and Gensini score in the UAP group. UAP: unstable angina pectoris; INF- γ : interferon- γ .**

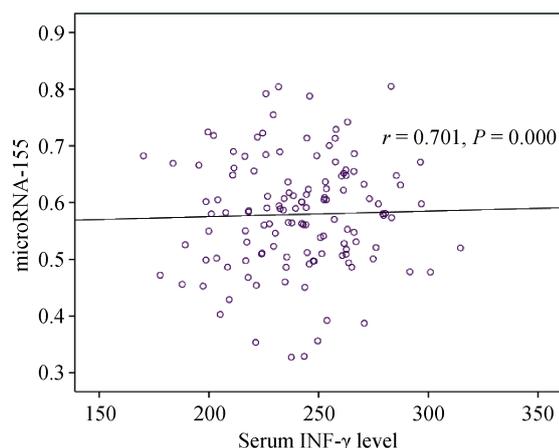


Figure 4. **Association between miRNA-155 and serum INF- γ level in the UAP group.** UAP: unstable angina pectoris; INF- γ : interferon- γ .

to rupture and CD4⁺ T lymphocytes are involved during the formation of inflammatory cells in unstable plaques.^[21,23] The activation of CD4⁺ T lymphocytes not only release IFN- γ , activation of monocytes and other inflammatory cells, but also through the antigen dependent or non-dependent pathways to induce apoptosis of vascular smooth muscle cells and direct dissolution of endothelial cells, participating in the formation of unstable plaque.

MiRNA is a highly conserved non-coding small fragment of RNA,^[24,25] which can interact with mRNA to induce translational repression or RNA degradation, and thereby regulating gene expression. Studies have shown that miRNA exists in a specific tissue or cell, and can be released into a variety of biological fluids.^[26,27] A large body of evidence suggests that miRNA play an important role in the abnormal expression of blood in patients after myocardial infarction^[28] and heart failure.^[29] MiR-155, a subtype of the miRNA family, play an important role in maintaining the function of T lymphocytes. In our previous studies,^[30] we found that the levels of CD4⁺ T lymphocyte miRNA-155 in patients with UAP were significantly higher, and miRNA-155 was involved in the differentiation, activation and functional regulation of CD4⁺ T lymphocyte and its subsets. The results of this study showed that the levels of CD4⁺ T lymphocyte miRNA-155 in the UAP group were significantly higher than that of the control group. According to the results of coronary angiography, the expression of miRNA-155 increased with the severity of coronary artery disease. Furthermore, the expression level of miRNA-155 was positively correlated with the Gensini score of coronary artery lesions, which indicated that the levels of CD4⁺ T lymphocyte miRNA-155 was closely related to the occur-

rence of coronary heart disease, especially have a close relationship with the occurrence of ACS.

It was found that a large number of activated IFN- γ in ACS patients not only decreased the proliferation of smooth muscle, but also produced collagenase, degraded and weakened the fibrous cap, which made the plaque rupture easily.^[31] In our study, we also found that the IFN- γ concentration in patients with UAP was significantly increased. Besides, with the severity of coronary artery disease, the concentration of IFN- γ was gradually increased. The correlation analysis showed that the IFN- γ concentration was positively correlated with the Gensini score of coronary artery lesion, which indicated that IFN- γ had an important significance in the occurrence and development of unstable coronary plaque. In addition, the correlation analysis of miRNA-155 and INF- γ showed that there was a significant positive correlation between the level of CD4⁺ T lymphocyte microRNA-155 and the concentration of serum INF- γ . The results showed that the miRNA-155 level of CD4⁺ T lymphocytes in the patients with UAP was correlated with the expression of serum INF- γ , which suggested that the expression of INF- γ was associated with miRNA-155. Our result is similar with some previous studies.^[32] Banerjee found that promoting the expression of miRNA-155 in CD4⁺ T lymphocytes could increase the concentration of INF- γ , and the effect may be achieved by negative regulation the expression of its target gene (IFN-cRa). In addition, Lu, *et al.*^[33] also found that the levels of miRNA-155 in CD4⁺ T lymphocytes was increased in patients with unstable angina pectoris, which was in agreement with our results. However, the results of our study are different from some previous studies. Zhu, *et al.*^[34] found that microRNA-155 is inversely associated with severity of coronary stenotic lesions calculated, which was contrary to the results of this study. The possible explanation is as follows. In the study conducted by Zhu, *et al.* 110 patients were included. However, 252 patients were recruited in our study, which means that the number of patients is more than two times that than of previous studies, thus leading to a different statistical efficacy and a different result.

Overall, the level of CD4⁺ T lymphocyte microRNA-155 and the concentration of serum INF- γ increased with the increase of severity of coronary artery disease. These results suggest that miRNA-155 and INF- γ are the adverse factors of coronary heart disease, which are closely related to the instability of plaque. The detection of the level of CD4⁺ T lymphocyte microRNA-155 may be a valuable routine method to predict the vulnerable plaques in patients with coronary heart disease, which should be paid more attention

to in clinical practice. In application, whether to reduce the morbidity of coronary heart disease, especially the incidence of ACS through reducing the expression of miRNA-155, still needs further extensive basic and clinical studies to be confirmed.

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