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## Expression level of miR-155 in peripheral blood

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### ABSTRACT

**Objective:** To investigate the relationship between the expression level of miR-155 and the severity of coronary lesion, and explore the action mechanism. **Methods:** Peripheral blood mononuclear cells (PBMC) were isolated from blood sample from patients with acute myocardial infarction (AMI), unstable angina (UAP), stable angina (SAP) and chest pain syndrome (CPS). RT-PCR was performed to analyze the expression level of miR-155 in peripheral blood mononuclear cells, plasma and RAW264.7 macrophagocyte. MTT was used to analyze the cell viability of OxLDL treated RAW264.7 macrophagocyte. **Results:** The expression level of miR-155 in blood sample from coronary heart disease patients was much lower than in the blood sample of non-coronary heart disease ( $P < 0.05$ ). The level of miR-155 in PBMCs was much higher in the blood sample from CPS group than the other three group, and the level of miR-155 in plasma was higher in the CPS group than in the UAP and the AMI group, the difference was statistically significant ( $P < 0.05$ ). The expression level of miR-155 in PBMCs is positively associated with the level in the plasma ( $r = 0.861$ ,  $P = 0.000$ ). OxLDL can induce the expression of miR-155 in RAW264.7 macrophagocyte, decrease the cell viability of RAW264.7 macrophagocyte, and with the concentration and the treatment time of OxLDL increased, the effect become more obvious. The inhibition effect of OxLDL to RAW264.7 macrophagocyte with high miR-155 expression is much lower than the control group, and it is statistically significant after treated for 12, 24 and 48 h. **Conclusions:** miR-155 plays a protective role in the progression of atherosclerosis, and it may be achieved by reducing the apoptosis effect of OxLDL to RAW264.7 macrophagocyte.

## 1. Introduction

MiR-155 is widely expressed in various cells, such as T cells, B cells, mononuclear cells and endothelial cells. As a multifunction miRNA, it is extensively involved with the differentiation, proliferation and apoptosis of many cells, and the development

of many tissues[1]. MiR-155 can inhibit inflammatory response and affect lipid uptake in macrophages through regulating other targets[2,3]. CAD is a kind of inflammatory disease closely related to lipid metabolism. There are no obvious symptoms during the early stage of CAD. However, as stable plaque develops into a vulnerable plaque, the fibrous cap may rupture and expose the liquid in the plaque, which could activate the fibrinolytic system in circulation. Eventually, occlusive thrombus may form, causing myocardial infarction[4–6]. Stephan Fichtlscherer *et al*[7] measured the miR-155 levels of patients with CAD and healthy patients; the results showed that miR-155 levels clearly declined for patients with CAD,

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compared with healthy patients. Huang *et al*[8] found a marked increase in the protein amount of the myeloid differentiation primary response gene 88 (MyD88), which can activate the NF- $\kappa$ B pathway. The results demonstrated that miR-155 serves as a negative feedback regulator in oxLDL-stimulated THP-1 inflammatory responses and lipid uptake. These phenomena suggest that miR-155 may be closely related to the disease. However, the relationship of miR-155 with the severity of the disease and the action mechanism is still unclear.

To investigate the clinical value of miR-155 in the diagnosis of CAD, we detected the miR-155 expression levels in plasma and mononuclear cells of peripheral blood from patients with different severities of CAD, analyzed the effort of miR-155 on the apoptosis of macrophage; the correlation of miR-155 with the severity of CAD.

## 2. Materials and methods

### 2.1. Materials

miR-155 mimics and miR-155 inhibitor were purchased from the Gene Pharma; OxLDL was purchased from the Beijing Xiessheng biological technology; DMEM culture medium and fetal calf serum were provided by HyClone; trypsin and MTT were from Sigma; total RNA isolation kit and First Strand cDNA Synthesis kit were from TaKaRa Bio Inc. Real-time PCR was purchased from Thermo; microplate reader was from Molecular Devices.

### 2.2. Cell isolation

The blood sample was provided by Shanghai East hospital. There were four kind of blood samples, namely blood from patients with acute myocardial infarction (AMI group), patients with tunstable angina pectoris (UAP group), patients with stable angina pectoris (SAP group), and patients with chest pain syndrome (CPS group). PBMCs were isolated from the blood by density gradient centrifugation method using lymphocyte isolation liquid. After centrifugation, the liquid divided into 3 layers; cells on the top of the forth layer were collected into a centrifuge tube, and was re-suspended by PBS. The liquid was centrifuged in 1 500 rpm for 15 minutes, then the supernatant was abandoned. The process was repeated for three times.

### 2.3. Transfect macrophage with miR-155 OligoRNA

RAW 264.7 was seeded in the 24 plate ( $2 \times 10^5$ /well), and cultured

to 80% confluence for later use. Lipofectamin 2000 ( $1 \mu\text{L}$ /well) and FAM-siRNA ( $2 \mu\text{L}$ /well) were diluted by  $50 \mu\text{L}$  Opti-MEM Reduced Serum Medium respectively, and cultured for 5 min; Mix the solution thoroughly and let it stand for about 20 min before using it. The FAM-siRNA-transfection reagent was added into the plate with culture medium, and after cultured in an incubator at  $37^\circ\text{C}$  for another 6 h, the liquid was replaced with DMEM culture medium containing 10% FBS. The cell was then collected and the expression level of miR-155 was analyzed by quantitative real time PCR.

### 2.4. Quantitative real-time PCR analysis

Certain amount of cell was re-suspended by PSB in a centrifuge tube, and an appropriate amount of Trizol was added into the centrifuge tube with the PBMCs. After incubating for 15 h the tube was centrifuged at 12 000 rpm at  $4^\circ\text{C}$  for 5 min, and the supernatant was removed. Total mRNA was isolated using an Eastep Universal RNA Extraction Kit (Promega). The RNA sample were treated with DNase I (Sigma), then quantified and reverse-transcribed into cDNA using a First Strand cDNA Synthesis kit (TAKARA). Quantitative real-time PCR was conducted using a RealPlex4 real-time PCR detection system (Eppendorf) with SYBR Green Real time PCR Master MIS (TOYOBO). The reaction system: SYBR Premix Ex Taq ( $2 \times$ )  $12.5 \mu\text{L}$ , PCR Forward Primer ( $10 \mu\text{M}$ )  $1 \mu\text{L}$ , PCR Reverse Primer ( $10 \mu\text{M}$ )  $1 \mu\text{L}$ , DNA template  $2 \mu\text{L}$ ,  $\text{dH}_2\text{O}$   $8.5 \mu\text{L}$ . Reaction parameters: pre denaturation at  $94^\circ\text{C}$  for 30 s, denaturation at  $95^\circ\text{C}$  for 15 s, anneal at  $60^\circ\text{C}$  for 20 s, and extend at  $72^\circ\text{C}$  for 10 s. The application was performed for 40 cycles. A comparative threshold cycle ( $C_t$ ) was used to determine the relative gene expression normalized to 18S rRNA for each sample, and the relative expression level of each sample was calculated using the formula,  $2^{-\Delta\Delta C_t}$ . All primers used in our study were designed according to the sequence published online (GenBank), and synthesized by Invitrogen.

### 2.5. MTT detect the viability of the cell

The RAW264.7 macrophage and miR-155 overexpressed RAW264.7 macrophage were digested by trypsin after they achieved 80% confluence. Adjust the concentration of the cell suspension to  $2 \times 10^5$ /mL, and  $200 \mu\text{L}$ /well was added into the 96 well plate. After cultured for 24 h, OxLDL was added to one group of the cell with the final concentration of 0, 20, 40, 80, 160  $\mu\text{g}/\text{mL}$ . the cell was cultured in an incubator at  $37^\circ\text{C}$  for another 4 h, and then the culture medium with MTT was abandoned,  $150 \mu\text{L}$  dimethyl sulfoxide (DMSO) was added to the plate to dissolve the formazan crystals

for 30 min. Then the absorbance was measured at 490nm using a Microplate reader. Corrected absorbance values were obtained by subtracting the blank absorbance from the absorbance obtained. OxLDL was added to the other group with the final concentration of 80  $\mu$ g/mL, after cultured for 1, 3, 6, 12, 24, 48 h, detect the absorbance as we described above.

## 2.6. Statistical analysis

In this study, SPSS 13.0 software was used for statistical analysis. The measured data were expressed as mean $\pm$ SD, and *t*-test was applied to the results for statistical analysis. The *chi*-square test was applied to the count data for statistical analysis.  $P < 0.05$  was considered significant.

## 3. Results

### 3.1. Levels of miR-155 in PBMCs and miR-155

Figure 1 shows the same trend of miR-155 expression levels between plasma and PBMCs, in the four kind of blood sample. The miR-155 levels declined as the disease become more severe: the highest miR-155 level came from the CPS group, followed by the SAP and UAP groups, and the AMI group had the lowest expression level. In PBMCs, the miR-155 levels were much higher than the other three groups, and the difference was statistically significant ( $P < 0.05$ ). In plasma, there were no significant differences between the miR-155 levels of the CPS and SAP groups ( $P > 0.05$ ), however, the miR-155 levels of the CPS group were obviously higher than the UAP and AMI groups ( $P < 0.05$ ).

In PBMCs, the miR-155 level in the CPS group was higher than in the SAP, UAP, and AMI groups; the differences were statistically significant (CPS *vs.* SAP  $t = 28.891$ ,  $P = 0.000$ ; CPS *vs.* UAP  $t = 20.739$ ,  $P = 0.000$ ; CPS *vs.* AMI  $t = 26.118$ ,  $P = 0.000$ ). In plasma, the miR-155 level in CPS was higher than in the UAP and AMI groups; the differences were statistically significant (CPS *vs.* SAP  $t = 10.065$ ,  $P = 0.167$ ; CPS *vs.* UAP  $t = 10.774$ ,  $P = 0.005$ ; CPS *vs.* AMI  $t = 15.036$ ,  $P = 0.000$ ).

### 3.2. miR-155 levels in PBMCs and plasma

Spearman correlation coefficients were used in evaluating and analyzing the correlation of miR-155 expression levels in PBMCs and plasma; and the results revealed that miR-155 levels has a positive correlation with PBMCs and plasma ( $r = 0.861$ ,  $P = 0.000$  (Figure 2).

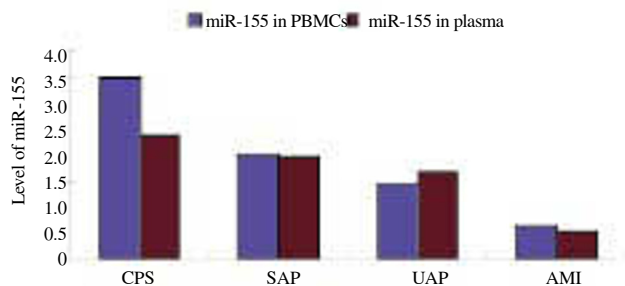


Figure 1. MiR-155 expression levels in PBMCs and plasma.

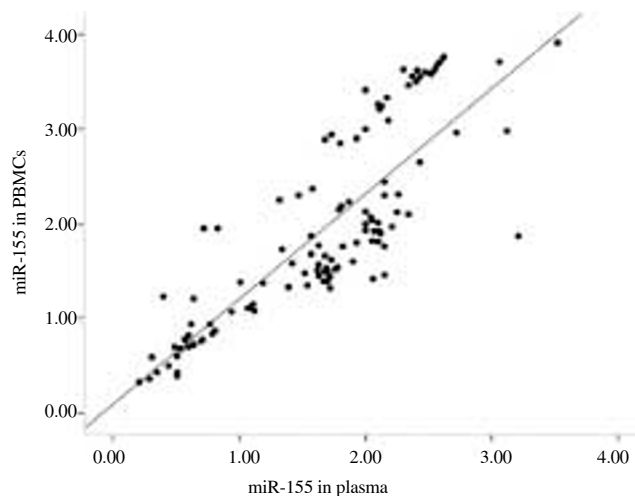


Figure 2. Correlation of miR-155 levels with PBMCs and plasma.

### 3.3. miR-155 levels between CAD blood sample and non-CAD blood sample

Patients were diagnosed with CAD when the degree of coronary stenosis was higher than 50%. As shown in figure 3, the miR-155 level in PBMCs was higher in the non-CAD group than in the CAD group ( $2.705 \pm 0.310$  and  $0.983 \pm 0.220$ , respectively); and the miR-155 level in plasma was also higher in the non-CAD group than in the CAD group ( $2.060 \pm 0.410$  and  $1.128 \pm 0.250$ , respectively).

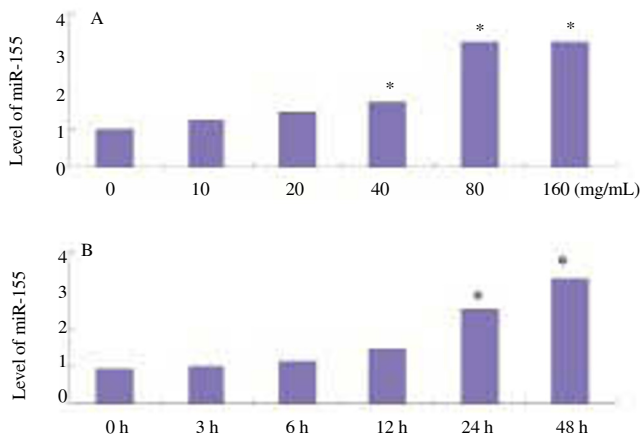
In PBMCs and plasma, the miR-155 levels in CAD patients were lower than non-CAD patients, and the difference was statistically significant (In PBMCs: non-CAD *vs.* CAD  $t = 11.550$ ,  $P = 0.000$ ; in plasma: non-CAD *vs.* CAD  $t = 14.003$ ,  $P = 0.002$ ).

### 3.4. Expression of miR-155 was induced by OxLDL by RAW264.7 macrophage

The level of miR-155 was detected by Real-Time PCR. We found that, after treated with OxLDL, the expression level of miR-155 in RAW264.7 macrophage raised with the improvement of the OxLDL concentration (Figure 3).

### 3.5. OxLDL reduce the viability of RAW264.7 macrophage

After treated with OxLDL of different concentration for 24 h, the viability of RAW264.7 macrophage declined with the concentration improved. Compared with the control group, the viability of the cell treated with the concentration of 20, 40, 80 and 160  $\mu$ g/mL declined obviously, and the difference was statistically significant ( $P<0.05$ ). When treated with OxLDL at the concentration of 80  $\mu$ g/mL, the viability of the cell also declined with the time prolonged. Compared with the control group, the viability of the cell at 24 and 48 hours improved significantly ( $P<0.05$ ) (Figure 4).



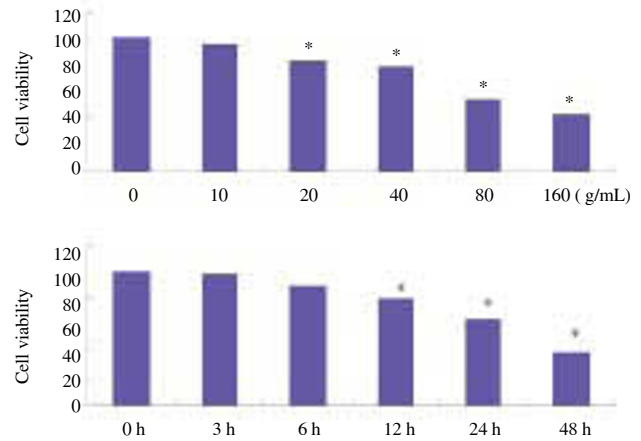
**Figure 3.** OxLDL induce the expression of miR-155 in RAW264.7 macrophage.

A: as the concentration of OxLDL incr RAW264.7 macrophage eased, the expression level of miR-155 in RAW264.7 macrophage improved; compared with the control group,  $*P<0.05$ . B: treated RAW264.7 macrophage with OxLDL with the concentration of 80  $\mu$ g/mL, the expression level of miR-155 improved as the treatment time prolonged; compared with the control group,  $*P<0.05$ .

### 3.6. Effort of OxLDL on the miR-155 overexpressed RAW264.7 macrophage

After treated with OxLDL, the viability of the cell in the control group, miR-155 overexpression group, and the miR-155 overexpression + miR-155 inhibitor group declined with the treatment time prolonged. And the viability of the cell in miR-

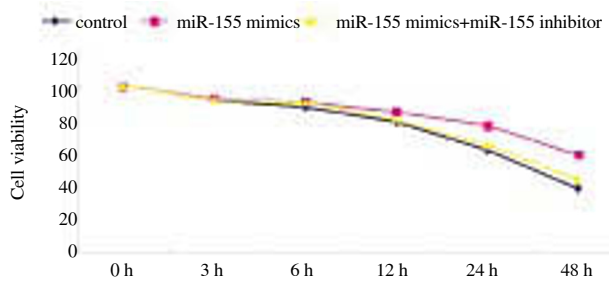
155 overexpression group was much higher than the control group and the inhibitor group in the 12, 24 and 48 h, the difference is statistically significant ( $P<0.05$ ). There was no statistical difference between the control group and the inhibitor group ( $P>0.05$ ) (Figure 5).



**Figure 4.** OxLDL reduce the viability of RAW264.7 macrophage.

A: with the concentration of OxLDL improved, the viability of RAW264.7 macrophage declined gradually; compared with the control group,  $*P<0.05$ .

B: after treated with OxLDL with the final concentration of 80  $\mu$ g/mL, the viability of the RAW264.7 macrophage declined with the treatment time prolonged; compared with the control group,  $*P<0.05$ .



**Figure 5.** Viability of miR-155 overexpression RAW264.7 macrophage after treated with OxLDL.

After treated with OxLDL, the viability of the cell in the three group were all declined with the treatment time prolonged. Compared with the control group, the viability of the cell in the inhibitor group declined obviously at 12, 24, 48 h, while there was no statistical significance between the control group and the miR-155 overexpression group. Compared with the control group,  $*P<0.05$ .

## 4. Discussion

miR-155 is a key factor that regulates inflammatory reactions and participates in inflammatory related diseases, while CAD is a kind of inflammatory disease; To elucidate the relationship of miR-155 with CAD, we measured the PBMCs and plasma miR-155 levels of blood sample form patients with CPS, SAP, UAP, and AMI; and analyzed

the relationship of miR-155 with the severity of CAD and plaque stability; detected the effort of OxLDL on RAW264.7 macrophage and miR-155 overexpression RAW264.7 macrophage, analyzed the effort of miR-155 on the apoptosis of RAW264.7 macrophage, these results further confirmed that miR-155 levels declines as CAD progresses and the overexpression of miR-155 can inhibit the apoptosis of RAW264.7 macrophage induced by OxLDL.

The miR-155 gene is located in chromosome 21q21, a highly conserved coding region within the third exon of the B-cell integration cluster gene[9,10]. Research revealed that miR-155 has been found up-regulated in several activated immune cells; which could modulate the immune response by regulating the differentiation of the immune cells, such as Th1 cell and T cell, and the secretion of cytokines[11,12]. Apart from inflammatory stimulation, the Oxidized LDL, which plays an important role in atherosclerosis, can also stimulate the THP-1 macrophage, and up-regulate the miR-155 level, which suggests that miR-155 plays an important role in CAD[13,14]. Fichtlscherer *et al*[15] found that miR-155 levels declines, as CAD progresses; while Menno Hoekstra *et al*[16] reported that there was no significant difference between the patient's miR-155 levels and the different degrees of CAD.

Our results showed that the miR-155 levels in plasma and PBMCs gradually declined from SAP to AMI blood sample. According to the correlation analysis, miR-155 levels in plasma were positively related to the levels in PBMCs. Although, the process on how miR-155 is released in blood circulation is still unclear, it can still be detected in serum, at a steady form[17]. This phenomenon revealed that miR-155 can resist RNA enzyme dependent degradation. During cell culture, miR-155 secretion can be stimulated by serum elimination[18,19]. Moreover, miR-155 can also be detected in endothelial cell-derived apoptotic bodies; and the RNA, incorporated into apoptotic bodies or microvesicles, can be delivered into recipient cells[20]. Combined with the results of our study, wherein, miR-155 can be detected in peripheral blood and that miR-155 levels in plasma has the same trend as the levels in PBMCs, we can deduce that miR-155 in plasma may have originated from PBMCs.

There was an over expression of miR-155 in the plaque and macrophage of patients with atherosclerosis[21,22], which suggesting that miR-155 could participate in the development of atherosclerosis. Although, miR-155 could accelerate inflammatory reactions and cell apoptosis at the same time[23], its function in atherosclerosis is still controversial. In our study, we found that the miR-155 levels in blood sample from patients with CAD were lower than blood sample from patients without CAD. This suggests that the miR-155 levels were negatively related to the severity of coronary lesions. Combined with the research of Li *et al*[24,25], we can speculate that miR-155 has a vascular protective effect; miR-155 can control inflammation and reduce tissue damage through its negative feedback effects on inflammatory factors. Moreover, miR-155 can also directly act on

angiotensin II -1 receptors, preventing it to bond with angiotensin II; which may block endothelial cell migration, repress angiotensin II -1 transcription, and inhibit the occurrence and development of atherosclerosis[26,27].

The improvement of apoptosis *in vivo* is a major cause of CAD, but the action mechanism is still unclear. The apoptosis of macrophage has been confirmed to be an outstanding factor in advanced atherosclerotic plaque. In this study, we use miR-155 mimics as intervention factor, induce the apoptosis of macrophage by OxLDL to stimulate the apoptosis process of macrophage *in vivo*. We found that OxLDL can induce the apoptosis of macrophage in an time and dosage dependent way. We also found, OxLDL can induce the expression of miR-155 improved slightly. The overexpression of miR-155 can improve the viability of macrophage, inhibit the apoptosis induced by OxLDL and the phenomena can be inhibited by miR-155 inhibitor. These results suggest that the regulation effort of OxLDL and miR-155 on the apoptosis of macrophage is achieved by a negative feedback pathway. Zhu *et al*[28] reported, miR-155 suppress the apoptosis of macrophage by regulate FADD.

In conclusion, the miR-155 levels of CAD patients were higher than non-CAD patients, and the miR-155 levels declined as the disease developed. The over expression of miR-155 can inhibit the apoptosis of macrophage induced by OxLDL. Therefore, the miR-155 in circulation may potentially serve as a new marker for cardiovascular disease. However, our study still has its deficiencies. For inflammatory related small miRNAs, it is still unclear whether other inflammatory diseases would affect the expression level of miR-155, and further affect the sensitivity and specificity of CAD diagnosis. Further study should be done to confirm the possibility of clinically displacing the tradition marker.

## Conflict of interest statement

We declare that me have no conflict of interest.

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