

The expression level of miR-155 in plasma and peripheral blood mononuclear cells in coronary artery disease patients and the associations of these levels with the apoptosis rate of peripheral blood mononuclear cells

DUO ZHAO^{1,2}, JING ZHAO², JIANGBIN SUN³, YANLING SU², JINFENG JIAN², HUAAN YE²,
JIAWANG LIN², ZONGDA YANG², JIATAO FENG² and ZHIPING WANG¹

¹Department of Cardiovascular Surgery, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510080; ²Department of Cardiovascular Surgery, The First People's Hospital of Foshan (Affiliated Foshan Hospital of Sun Yat-sen University), Foshan, Guangdong 528000; ³Department of Cardiovascular Surgery, Affiliated Hospital of Guilin Medical University, Guilin, Guangxi 541001, P.R. China

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Abstract. The aim of the study was to investigate the expression of miR-155 in plasma and peripheral blood mononuclear cells (PBMCs), the effects of miR-155 on the apoptosis rate of PBMCs and the extent of coronary stenosis in coronary artery disease patients. Seventy chest pain patients were divided into three groups by symptoms, signs, auxiliary examination and coronary arteriography: 21 cases in the acute myocardial infarction group (AMI), 23 cases in the Angor pectoris group (AP) and 26 cases in the control group (CT). The peripheral blood mononuclear cells of the patients in the three groups were separated for cell culture. Annexin V/propidium iodide (PI) was performed to analyze cell apoptosis. RT-qPCR was used to evaluate the expression level of miR-155 in plasma and PBMCs. There were significant differences on the expression of miR-155 in plasma and PBMCs, the apoptosis rate of PBMCs, Gensini score and the extent of coronary stenosis among the three groups ($P < 0.05$). The expression of miR-155 in plasma and PBMCs in AMI and AP group were lower than CT group while the AMI group was lower than the AP group

($P < 0.05$). The apoptotic rate of PBMCs, Gensini score and the extent of coronary stenosis of the AMI and AP groups were higher than CT group while the AMI group was higher than the AP group ($P < 0.05$). The expression of miR-155 in plasma was positively correlated with PBMCs, but negatively correlated with the apoptosis rate of PBMCs, Gensini score and the extent of coronary stenosis. The apoptosis rate of PBMCs in patients with coronary heart disease was positively correlated with the degree of coronary artery stenosis and Gensini score. In conclusion, in the patients with coronary heart disease the apoptosis rate of PBMCs was increased and the expression of miR-155 in plasma and PBMCs cells was decreased, which were correlated with the severity of coronary heart disease.

Introduction

Coronary heart disease is a common disease, involving coronary stenosis or occlusion of the lumen, triggering a series of symptoms, which poses a threat to the patient's life and health. Coronary heart disease is closely related to inflammation and immune and plays a role in the occurrence and development of coronary heart disease. The pathological basis of coronary heart disease is atherosclerosis, which is formed by long-term vascular inflammation and fibrosis together (1). Previous findings showed that there were autoimmune cell imbalance and immune function decrease in coronary heart disease patients (2).

miRNAs, as a research hotspot, are evolutionarily highly conserved, endogenous single-stranded, non-coding small molecule RNAs, which can regulate the expression of protein-coding genes after transcription. A large number of studies have proved that miRNAs play an important role in cell differentiation, apoptotic cells, angiogenesis, lipid metabolism, inflammation, immune and other physiological and pathological process (3-5). miR-155 is located on human chromosome 21 and plays an important role in hematopoiesis, immunity, inflammation, cancer and cardiovascular

Correspondence to: Dr Zhiping Wang, Department of Cardiovascular Surgery, The First Affiliated Hospital of Sun Yat-sen University, 58 Zhongshan Road, Guangzhou, Guangdong 510080, P.R. China

E-mail: wangzhipingcc@126.com

Dr Jiatao Feng, Department of Cardiovascular Surgery, The First People's Hospital of Foshan (Affiliated Foshan Hospital of Sun Yat-sen University), 81 North South of the Five Ridges Avenue, Foshan, Guangdong 528000, P.R. China

E-mail: surgeonzhaodao@qq.com

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disease (6-8). A previous study found that miR-155 was closely related to immune inflammatory response in the process of atherosclerosis (9,10). Peripheral blood mononuclear cells (PBMCs) are mononuclear leucocytes isolated from peripheral blood, which is composed of T and B lymphocytes, macrophages, and natural killer cells (NKs) and plays a crucial role in the immune response (11).

The aim of the current study was to observe the changes of miRNA-155 in plasma and PBMCs of coronary heart disease patients and to investigate the relationship of miRNA-155 with the apoptosis rate of PBMCs and the severity of coronary arteries, in order to provide the basis for further study on the effect of miRNA-155 in coronary heart disease.

Materials and methods

Seventy cases with chest pain were randomly selected from January 2014 to December 2016 in the Department of Cardiology in The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). The data and venous blood were collected and 3 groups were separated by clinical symptoms and signs, laboratory examinations and coronary angiography results as referenced in the Chinese Medical Association Cardiovascular Branch standards. There were 21 cases of acute myocardial infarction (AMI group: history of typical chest pain, dynamic changes of electrocardiogram, elevated myocardial enzymes and troponin, at least one vessel stenosis or occlusion of coronary angiography), 23 cases in the angina pectoris group (AP group, chest pain history, ECG changes, rest or medication relief, coronary angiography to determine coronary artery stenosis) and 26 cases in the control group (CT group, chest pain but no coronary lesions).

All the patients enrolled in this study were first-episode and the following diseases were excluded: Atrial fibrillation, pacemaker implantation, valvular heart disease, malignancy, thromboembolic disease, severe liver-kidney dysfunction and stroke. There were no recent infection and no immune-related history. The study was approved by the Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University and the patients signed informed consent form. The time of blood collection from all the patients was within 24 h after chest pain attack.

Coronary angiography and Gensini score (9): The coronary angiography of all the cases were carried out by the same group of doctors through the radial artery or femoral artery approach, and all segments of the coronary arteries were fully displayed. Coronary lesions were quantitatively scored according to the coronary angiographic recording segment criteria set forth in the AHA (9), then the Gensini score and the degree of coronary stenosis were recorded.

Isolation of PBMCs. Venous blood (10 ml) was collected from all the patients in the early morning, which was anticoagulated by Heparin, and the Lymphocyte separation fluid was added. The blood was centrifuged at 1,600 x g for 20 min at 4°C and the cells were divided into four layers: First layer, plasma or tissue homogenate layer; second layer, cyan milky lymphocyte or monocyte layer; third layer, transparent separation layer; and fourth layer, red blood cell layer. The cells in the second layer were harvested and washed twice with 5 volumes of

normal saline prior to centrifugation at 1,600 x g for 10 min at 4°C. The cell pellet was resuspended in phosphate-buffered saline (PBS) and divided into two portions. One of the portions was added to a cell culture flask and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, with the cell concentration of 1x10⁶/ml, and the other was used for RNA extraction.

Apoptosis detection by Annexin V-FITC/propidium iodide (PI). PBMCs were seeded in 6-well plates and cultured in an incubator for 48 h. The cells were harvested and resuspended at a density of 1x10⁶/ml in binding buffer. The PBMCs were labeled by Annexin V-FITC and PI according to instructions of Annexin V-FITC/PI Dual Staining kit (Invitrogen, Carlsbad, CA, USA). Firstly, 5 µl Annexin V-FITC was added and incubated for 10 min in the dark. Then the tube was centrifuged, the supernatant discarded and the buffer re-added to resuspend. Secondly, 10 µl PI staining solution was added, mixed and dark-stained for 15 min at 4°C. Finally, the samples were detected by flow cytometry (cytometry; Beckman Coulter, Brea, CA, USA). Experiments were repeated at least three times.

Detection of miR-155 in plasma and PBMCs. Peripheral blood (6 ml) was collected in EDTA anticoagulant tube, with the patients fasting 8 h, prior to centrifugation at 1,600 x g for 10 min at 4°C twice. Supernatant of 500 µl was collected as a plasma sample. Total RNA was extracted from the plasma sample and PBMCs cells using TRIzol LS reagent according to the manufacturer's protocol and stored at -80°C for reverse transcription. Total RNA (20 ng) from each sample was used for reverse transcription according to the manufacturer's protocol of the TaqMan microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Primers used for the TaqMan microRNA assays (Applied Biosystems; Thermo Fisher Scientific) were: Control U6 primers, upstream, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and downstream, 5'-CGCTTCACGAATTTGCGTGTTCAT-3'. miRNA-155 primers, upstream, 5'-GGAGGTTAATGCTAATCGTGATAG-3' and downstream, 5'-GTGCAGGGTCCGAGGT-3'.

The reaction conditions were as follows: 95°C for 15 sec and 40 cycles of 60°C for 30 sec. We repeated each sample in each group in triplicate. Reverse transcription product (2 µl) was used for TaqMan probes reverse transcription-quantitative polymerization chain reaction (RT-qPCR) and PCR double-specific primers were amplified using the TaqMan universal Master Mix II, no UNG (Applied Biosystems; Thermo Fisher Scientific) by the ABI 7500 Thermal Cycler to detect Cq value of the target gene and control gene using 2^{-ΔΔCq} method according to the following formula: ΔΔCq (target gene) = Cq (target gene) - Cq (control gene) (12).

Statistical analysis. The experimental data were expressed as mean ± standard deviation and analyzed using SPSS 18.0 software. Student's t-test, and Chi-square test were used for comparisons between two groups. For pairwise comparisons ANOVA and LSD post hoc test were used. Spearman's linear correlation analysis were used to analyze the data. P<0.05 or P<0.01 indicated a statistically significant difference.

Table I. General information for patients.

Group	No.	Age	Male/female	Hypertension	Diabetes	Hyperlipidemia	Smoke	β -blocker	ACEI	CCB	Statins
AMI	21	59 \pm 7	15/6	13	5	13	6	14	15	9	17
AP	23	58 \pm 9	17/5	13	4	11	5	13	10	11	15
CT	26	60 \pm 8	16/10	10	3	7	5	7	8	8	10

AMI, myocardial infarction group; AP, Angor pectoris group; CT, control group.

Table II. miR-155, apoptosis rate of PBMCs, coronary artery stenosis and Gensini score in patients.

Group	No.	Apoptosis rate of PBMCs (%)	Degree of coronary artery stenosis (%)	Gensini score	miR-155 (plasma)	miR-155 (PBMCs)
AMI	21	34.3 \pm 5.4 ^{a,b}	87.61 \pm 8.78 ^{a,b}	49.15 \pm 12.87 ^{a,b}	0.69 \pm 0.10 ^{a,b}	0.56 \pm 0.08 ^{a,b}
AP	23	18.2 \pm 1.6 ^a	62.40 \pm 5.98 ^a	33.71 \pm 8.87 ^a	1.55 \pm 0.29 ^a	1.34 \pm 0.25 ^a
CT	26	2.7 \pm 1.22	16.6 \pm 9.4	0.70 \pm 1.95	3.00 \pm 0.80	2.32 \pm 0.53

^aP<0.05 vs. CT group; ^bP<0.05 vs. AP group. PBMCs, peripheral blood mononuclear cells; AMI, myocardial infarction group; AP, Angor pectoris group; CT, control group.

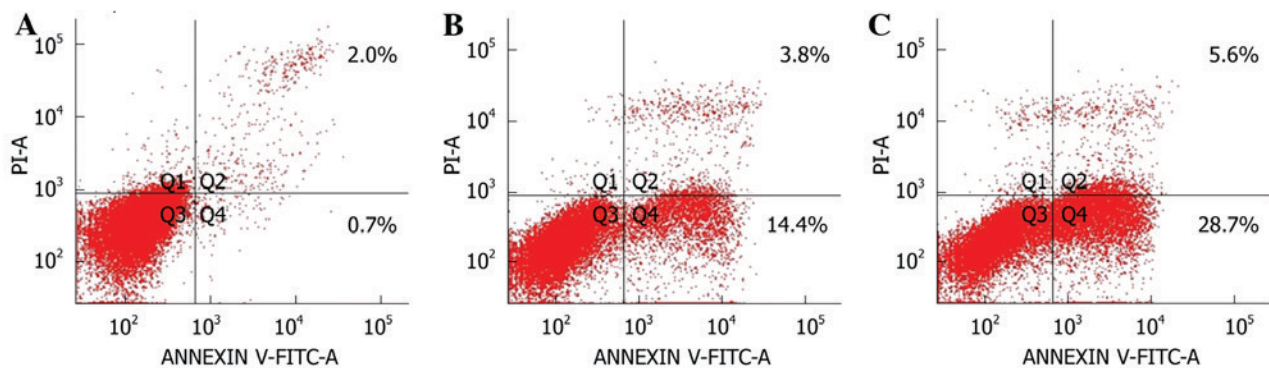


Figure 1. Apoptosis rate of peripheral blood mononuclear cells (PBMCs) in the (A) CT, (B) AP group, and (C) acute myocardial infarction (AMI) groups.

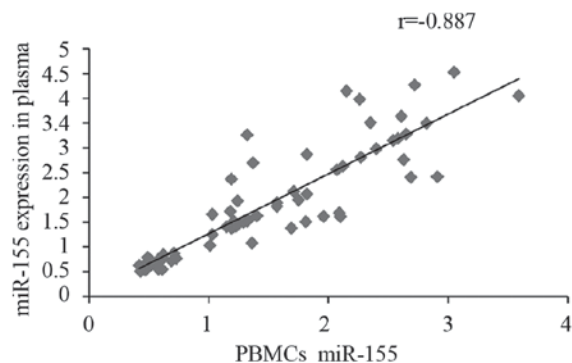


Figure 2. The correlation between miR-155 levels in plasma and PBMCs.

Results

General situation comparison. There was no statistically significant difference of age, sex, disease history and medication ($P>0.05$) among the patients in the three groups (Table I).

Apoptosis of PBMCs in each group. The apoptotic rates of PBMCs in the three groups were detected by flow cytometry: 34.3 \pm 5.4% in the AMI group, 18.2 \pm 1.6% in the AP group and 2.7 \pm 1.22% in the CT group. The apoptosis rate of PBMCs in the AMI group was significantly higher than that in the AP and CT groups ($P<0.05$). The apoptosis rate of PBMCs in the AP group was significantly higher than that in CT group, and the difference was statistically significant ($P<0.05$) (Fig. 1).

Coronary stenosis and Gensini score in each group. The degree of coronary artery stenosis and Gensini score in the AMI group were significantly higher than those in the AP and CT groups ($P<0.05$). The degree of coronary artery stenosis and Gensini score in the AP group were significantly higher than those in CT group ($P<0.05$) (Table II).

Comparison of miR-155 levels in each group. The level of miR-155 in AMI group was significantly lower than that in the AP and CT groups ($P<0.05$) and the level of miR-155 in AP group

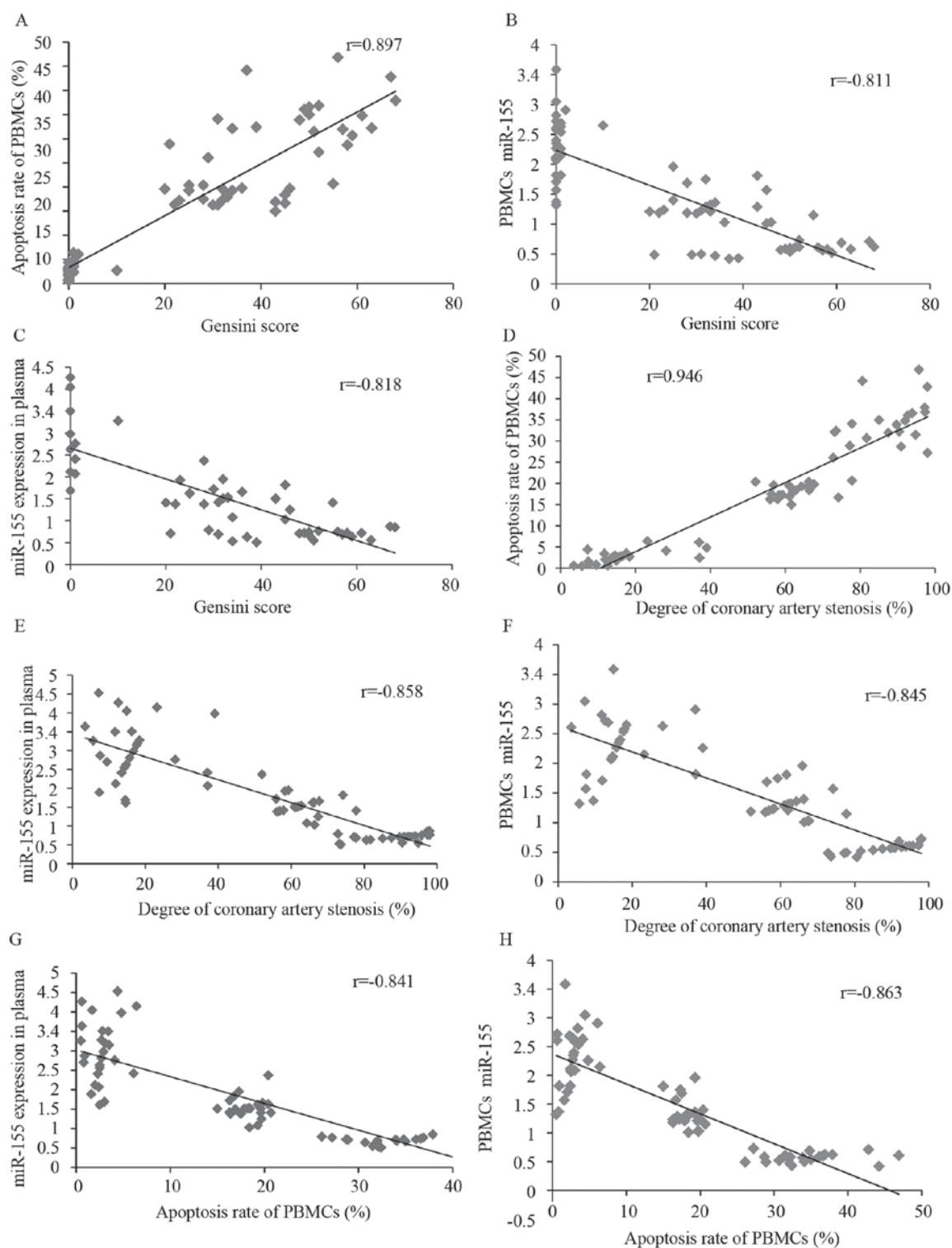


Figure 3. The correlation of miR-155, apoptosis rate of peripheral blood mononuclear cells (PBMCs) with coronary artery stenosis and Gensini score. The apoptosis rate of PBMCs in patients with coronary heart disease was positively correlated with the Gensini score (A) and the degree of coronary artery stenosis (D). The expression level of miR-155 in PBMCs of patients with coronary heart disease was negatively correlated with the Gensini score (B), the degree of coronary artery stenosis (F) and the apoptosis rate of PBMCs (H). miR-155 expression in plasma of patients with coronary heart disease was negatively correlated with the Gensini score (C), the degree of coronary artery stenosis (E) and the apoptosis rate of PBMCs (G).

was significantly higher than that in CT group ($P<0.05$). There was a positive correlation between miR-155 levels in plasma and PBMCs in three groups ($r=-0.887$, $P<0.001$) (Table II, Fig. 2).

Correlation of miR-155, apoptosis rate of PBMCs with coronary artery stenosis and Gensini score. As shown in Fig. 3,

miR-155 expression in plasma of patients with coronary heart disease was negatively correlated with the Gensini score, the degree of coronary artery stenosis and the apoptosis rate of PBMCs ($r=-0.818$, $P<0.001$; $r=-0.858$, $P<0.001$; $r=-0.841$, $P<0.001$). The expression level of miR-155 in PBMCs of patients with coronary heart disease was negatively correlated

with the Gensini score, the degree of coronary artery stenosis and the apoptosis rate of PBMCs ($r=-0.811$, $P<0.001$; $r=-0.845$, $P<0.001$; $r=-0.863$, $P<0.001$). The apoptosis rate of PBMCs in patients with coronary heart disease was positively correlated with the Gensini score and the degree of coronary artery stenosis ($r=0.897$, $P<0.001$; $r=0.946$, $P<0.001$).

Discussion

The pathogenesis of coronary heart disease is complicated. A variety of factors lead to the damage of intima, long-term vasculitic inflammation and fibrogenesis causing the formation of atherosclerotic plaque. Inflammatory reaction promotes the formation and development of atherosclerotic plaque, which plays an important role in the process of coronary heart disease (13). The Gensini score was used to assess the severity of coronary lesions by giving quantified weights of different sites of stenosis and the degree of stenosis. The higher the score, the more severe the coronary artery disease. This method has been widely used clinically to assess the severity Degree of coronary artery disease (9,14,15).

miR-155 is a multi-functional miRNAs located in human chromosome 21 and expressed in many tissues and cells, involved in many physiological and pathological processes such as immunity, inflammation, cell differentiation, cardiovascular diseases and tumors. miR-155 is closely related to inflammation and immunity, and can regulate the activation of immune cells and the release of immune factors (6-8). Studies have shown that miR-155 regulates the transcription of angiotensin II-1 receptors and affects the migration of endothelial cells and thus the progression of atherosclerosis (16). However, the current expression of miR-155 in coronary heart disease is controversial. Studies have shown that miR-155 is upregulated in atherosclerotic mouse models (17,18), but other studies have shown that miR-155 is significantly decreased in patients with coronary heart disease compared with non-CAD patients (9,10,19). The results in our study have shown that the expression of miR-155 in plasma and PBMCs in patients with myocardial infarction was significantly lower than that with angina pectoris. The expression level of miR-155 in patients with angina pectoris was significantly lower than that with non-coronary heart disease. Moreover, the expression of miR-155 in plasma and PBMCs was highly negatively correlated with the severity of coronary heart disease. This study is consistent with some studies (9-11,19).

It is generally accepted that atherosclerosis is an inflammatory disease, as abnormal activation of immune cells, excessive inflammatory mediators released, excessive inflammatory reactions, damage to coronary endothelial cells, causing platelet aggregation, thrombosis, which accelerated the formation of coronary atherosclerotic plaques, severe even porridge plaque rupture and induced myocardial infarction or acute angina attacks (20). Immune cells in patients with coronary heart disease repeatedly activated, apoptosis, resulting in decreased immune cells, impaired immune function, varying degree of decreased immune function in coronary heart disease patients. Previous findings have shown that miR-155 regulates the differentiation of T lymphocyte subsets by regulating the expression of two target genes of SMAD2 and SOCS1 in coronary heart disease (21). SMAD2

can induce the differentiation of Th17 cells and the production of interleukin-17A (IL-17A), while SOCS1 inhibition of Th17 cell differentiation by inhibiting IL-6/STAT3 signaling pathway (22), suggesting that miR-155 expression is negatively correlated with Th17 differentiation. However, little is known about the expression of miR-155 and the apoptosis of PBMCs. The present study has shown that the apoptosis rate of PBMCs in patients with myocardial infarction is significantly higher than that with angina pectoris, while the apoptosis rate of PBMCs in patients with angina pectoris is significantly higher than that in control group ($P<0.05$). The apoptosis rate of PBMCs is highly positive correlation with the severity degree of coronary heart disease, while the apoptosis rate of PBMCs and the miR-155 expression in plasma and PBMCs was negatively correlated.

In conclusion, the apoptosis rate of PBMCs in patients with coronary heart disease increased, and the expression of miR-155 in plasma and PBMCs decreased, which were all related to the severity of coronary heart disease.

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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

DZ and ZY performed RT-PCR. JZ, JS and YS were responsible for the isolation of PBMCs. JJ, HY and JL assisted in the apoptosis rate detection. JF and ZW contributed to statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) and the patients signed informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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