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The Association between Inflammatory Cytokines and miRNAs with Slow Coronary Flow Phenomenon

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ABSTRACT

Slow coronary flow (SCF) is a coronary artery disorder. Several inflammatory mediators have been reported to be associated with vascular homeostasis and endothelial dysfunction.

The aim of this study was to investigate the association between cytokines and miRNAs in patients with SCF compared to the controls. In this regard, blood samples were acquired from 45 SCF patients and 45 age- and sex-matched healthy control subjects. Serum and peripheral blood mononuclear cells (PBMCs) were separated. Expression levels of miRNAs and cytokines in PBMCs were measured by real-time PCR. As a final point, serum levels of cytokines were quantified by ELISA.

Expression levels of miR-1, miR-133, miR-208a, miR-206, miR-17, miR-29, miR-223, miR-326, and miR-155 as considerable indicators of inflammatory function significantly increased in SCF patients while the expression levels of miR-15a, miR-21, miR-25, miR-126, miR-17, miR-16 and miR-18a as considerable indicators of anti-inflammatory function significantly decreased in patients with SCF compared to the control group. Additionally, serum IL-1β, IL-8, and TNF-α concentrations were significantly higher in the SCF group than controls. However, no significant differences were observed in IL-10 production in SCF patients compared to the controls.

This study provided the potential role of miRNAs as biomarkers for SCF diagnosis as well as suitable markers for monitoring coronary artery disease (CAD) development in these patients. More investigations are still necessary to unravel the detailed essential mechanisms of circulating miRNA levels in patients with heart failure and SCF.

Keyword: Cytokines; Inflammation; miRNA; Slow coronary flow

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INTRODUCTION

Slow coronary flow (SCF) is a coronary artery disorder characterized by delayed opacification of the epicardial coronary arteries in the absence of coronary artery stenosis.[1,2] The prevalence of this common angiographic disorder has been reported as 1–7% in patients undergoing coronary angiography, 40% in patients with normal arteries, 16% in Cardiac Syndrome X (CSX) patients and 4% in patients experiencing angiography for fast assessment of unstable angina.[1] Risk factors for the SCF phenomenon are still unclear and studies carried out in different ethnic populations have found variable risk factors to be associated with the SCF phenomenon. Male sex, smoking, hyperuricemia, hyperglycemia, hypertension, thrombocytosis, high-sensitivity C-reactive protein (hs-CRP), and opioid abuse were found to be the independent risk factors for SCF.[3] Inflammatory mediators and cytokines have been reported to play a contributing role in some steps of atherosclerosis from local inflammation to plaque formation and rupture.[4] Pro-inflammatory cytokines such as Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-1 (IL-1) are important mediators released by inflammatory cells and result in endothelial cell dysfunction and cardiovascular diseases.[5] Plasma levels of total cholesterol and low-density lipoprotein (LDL-C) are also the main risk factors for coronary heart diseases.[6] Furthermore, IL-8 is a pro-inflammatory cytokine produced by numerous cell types including vascular endothelial cells, vascular smooth muscle cells and peripheral blood monocytes which are implicated in atherosclerosis.[7] Even though, the risk of emerging coronary slow flow is in reverse association with plasma high-density lipoprotein (HDL-C) levels.[8] In the heart tissue, miRNAs also modulate several myocardia physiological or pathological processes including cardiac development, cardiac remodeling, myocarditis, myocardial fibrosis, cardiomyocyte apoptosis which are detected in numerous cardiac disorders.[9] It has been revealed that miR-1 is involved in the development of heart and skeletal muscles and deregulated expression of miR-1 is implicated in cardiac disease pathogenesis.[10] Additionally, miR-133 and miR-208 come together round structures relating to myocardial tissue with contrary effects on cardiac hypertrophy, as miR-133 has both anti-fibrotic and anti-hypertrophic effects, whilst overexpression of miR-208 is pro-hypertrophic.[11] Evidence suggests that miR-208a is essential for cardiac remodeling promotion and arrhythmias, and modulates the expression of hypertrophy pathway components.[12] miR-21 also modulates the ERK–MAP kinase signaling pathway in cardiac fibroblasts and enhances their proliferation.[13] Members of the miR-29 family as a cardiac fibrosis modulator and a probable target of tissue fibrosis decrease in the district nearby to MI areas in mice and humans. miR-126 is also found to modulate the expression of Vascular Cell Adhesion Molecule 1 (VCAM1).[14] Recently, miR-21, miR-155, miR-126, miR-221, miR-223, and miR-29 are involved in vascular diseases, vessel remodeling, and inflammation.[15] The roles of miRNAs in inflammation or immune function are summarized in Table 1. Due to the high cost, invasive procedure, and test contradictions, there is a critical need to develop cost-effective, simple, and feasible alternative approaches for SCF diagnosis in early stages. Therefore, we investigated the relationship between cytokines and miRNAs in SCF patients and controls.

MATERIALS AND METHODS

Study Population

Forty-five patients aged 35 to 51 years referred to the Shahid-Madani Hospital of Tabriz University of Medical Sciences were enrolled from January to March 2018. SCF was diagnosed using an angiography technique and confirmed in all coronary arteries by a cardiologist. The randomly selected control group (n=45) also included subjects who experienced coronary angiography with a normal epicardial coronary artery angiogram. Inclusion criteria were coronary flow measured with TIMI frame count>23, compound angina pattern with electrocardiographic changes, and increased bleeding disorders incidence in scintigraphy. The use of nutritional supplements and immunosuppressive drugs, as well as pregnancy or lactation, autoimmune diseases, and other chronic infectious diseases were excluded from the study. Patients, who met the criteria, were then referred to the researcher and the study aims and methods were explained to the patients. Written informed consent was obtained from all patients before sampling. After completing the required questionnaires, 10 ml of intravenous blood

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Table 1. Role of miRNAs in inflammation or immune function

miRNAs	Function	References
miR-1	- Regulates the expression of GJA1 and SOX9 -Increased expression of TNF-α was associated with decreased expression of miR-1	(11)
miR-133	-Regulates myocardial collagen production by repressing TGF-β1 and TGF-β receptor type II expression - Endomyocardial miR-133 levels correlate with macrophage infiltration -Overexpression of miR-133: -Reduces ROS content -Increases SOD activity and GPx levels -Protects cardiomyocytes from apoptosis	(11)
miR-208a	- SOX6 was as a target of miR-208 -Overexpression of miR-208: - Increases cell proliferation -Tumorigenicity -Cell cycle progression - Overexpression of miR-208a up-regulates IL-10 secretion in human macrophages	(12)
miR-206	-NR4A2 is a target of miR-206 -Modulates LPS-induced inflammatory response -Increased expression of TNFα was associated with decreased expression of miR-206	(11)
miR-17	-Inhibits the expression of the PTEN and the pro-apoptotic protein Bim. -Up-regulated in lymphoproliferative diseases and autoimmunity. - Suppresses B cell development at the pro-B to pre-B transition	(36)
miR-29	-IFN-γ-inducible miRNA in CD4+ memory T cells that regulates Th1 cell bias by preventing from T-bet and IFN-γ transcription	(36)
miR-223	-Targets the transcription factor STAT5 and other inflammatory regulators such as Hsp90 and E2F	(30)
miR-326	-Stimulates Th17 differentiation by aiming Ets-1	(36)
miR-155	-Contributes to Th17 cell development -Targets HO-1, leading to increased T cell infiltration and enhanced chronic autoimmunity - Involves in proinflammatory signaling cascades and effector functions in macrophages	(36)
miR-15a	-Targets BCL-2, Cyclin D1, Cyclin D2 and MCL1 - Decreases the proliferation, migration, and invasion of immune cells by decreasing the expression of NF-κB	(37)
miR-16	-miR-16-1 targets and represses the expression of BCL-2 -Decreased proliferation of T cells	(32)
miR-21	-Is critical for T cell homeostasis -High expression in effector T cells	(13)
miR-25	-Contributes to the regulation of TGF-β signaling pathway by modulating the activity of the pro-apoptotic gene BCL2L11/Bim	(36)
miR-126	-Promotes angiogenesis and inhibits inflammation	(14)
miR-18a	-Target *Smad4*, *Hif1a*, and *Rora*, all key transcription factors in the Th17 cell gene-expression program - Inhibits Th17 differentiation	(38)

BCL-2, B-cell lymphoma 2; E2F, E2 factor; HO-1, heme oxygenase 1; Hsp90, heat shock protein 90; MCL-1, Induced myeloid leukemia cell differentiation protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells PTEN, phosphatase, and tensin homolog; ROS, reactive oxygen species; SOD, superoxide dismutase; STAT5, signal transducer and activator of transcription 5; TGF- β1, transforming growth factor β1; TNF-α, tumor necrosis factor-alpha.

were obtained from patients and the control group in a fasting state for making the same condition between participants in this study.

The present study was approved by the local research ethics committee of the Tabriz University of Medical Sciences (IR.TBZMED.REC.1396.1099).

Serum Separation

5 mL whole blood samples from the patients were collected into plain tubes, and serum was separated after centrifugation at 4000 rpm for 5 minutes. The serum was then stored at -80^{oC} for further analysis.

Serum Biochemical Markers Analysis

Serum total cholesterol, triglyceride (TG), HDL-C, low-density lipoprotein-cholesterol (LDL-C) and fasting blood sugar (FBS) levels were quantified by BT 3000 device using the test kit.

RNA Extraction and a Real-time Polymerase Chain Reaction

After 10 mL of whole blood samples collection, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by adding 1.077 g/mL Ficoll-Hypaque (lymphosep) (Biosera, UK) and centrifugation at 450×g for 25 min. Total PBMCs RNAs were extracted with Total RNA Purification Mini kit (YTA, Tehran, Iran) according to the manufacturer's procedure. Total RNA was quantified using a Nanodrop spectrophotometer (Agilent Technologies, USA). Consequently, complementary DNA (cDNA) was synthesized using 1 µg of total RNA, MMLV reverse transcriptase and oligo-dT primer according to the manufacturer's instructions (Thermo Fisher, Waltham, MA, USA). Relative gene expression was measured by real-time PCR using SYBR Premix Ex Taq (QIAGEN, Hilden, Germany) and the Light Cycler 2.0 Real-Time PCR System machine (Roche Applied Science, Germany). The standard condition for the SYBR Green technique in the first step was 10 min at 95^{o}C. This was repeated for 45 cycles of denaturation at 95ºC for 20 seconds and subsequently, annealing and extension were performed at 60ºC for 1 min. To evaluate the miRNAs expression, we analyzed 16 miRNAs expression from PBMCs samples including miR-1, miR-133, miR 208a, miR-206, miR-17, miR-29, miR-223, miR-326, miR-155, miR-15a, miR-21, miR-25, miR-126, miR-17, miR-16 and miR-18a by specific primers and SYBER Green Master Mix (QIAGEN, Hilden, Germany). Data were normalized according to the RNU6B and β-Actin expression and calculated using the relative quantification ($2^{-\Delta\Delta Ct}$) method. To investigate mRNA expression levels of cytokines, total RNA was isolated from PBMCs and cDNA was synthesized. The PCR reactions were done in 25 μL reaction volume comprising primers and the SYBR Green kit reagents (TaKara). All experiments were done in triplicate.

Enzyme-Linked Immune Sorbent Assay (ELISA)

Serum levels of TNF-α, IL-1β, IL-8, and IL-10 were evaluated by sandwich ELISA kit (Mybiosource, San Diego, USA) in duplicate. Briefly, monoclonal antibodies specific for TNF-α, IL-1β, IL-8, and IL-10 were pre-coated onto a microplate. Standards and samples were pipetted into the wells and any cytokine was bound by the immobilized antibody. Followed by washing, an enzyme-linked polyclonal antibody specific for these cytokines was added to the wells. After the final wash, a substrate solution was added to the wells and color development was stopped and the intensity of the color was measured. The absorbance values were quantified at 450 nm through a Medgenix ELISA reader (BP-800, Biohit, USA). Cytokine concentrations were calculated using the standard curves.

Statistical Analysis

Statistical analysis was done by SPSS PC Statistics (version 19.0; SPSS Inc.). Results were expressed as mean±standard deviation (SD). We used an unpaired t-test for normally distributed data between the controls and SCF patients. For drawing the graphs, GraphPad Prism (version 7.00 for Windows; GraphPad Software, La Jolla, CA; www.graphpad.com) was used. $p<0.05$ was considered as statistically significant.

RESULTS

Subject Characteristics

The clinical characteristics of all participants are shown in Table 2. Regarding the risk factors, diabetes, obesity, and smoking were reported in 9 (20%), 15 (33.3%), and 21 (46.7%) cases, respectively. No statistically significant differences were observed between SCF patients and the control group regarding the risk factors ($p>0.05$). Serum lipid profile analysis in SCF patients and the control group also showed a

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significantly higher total cholesterol in SCF patients compared to the control group (198.5±43.56 versus 172.5±54.3, p=0.02, respectively). Additionally, higher levels of TG, LDL-C, and FBS and lower levels of HDL-C were observed in SCF patients compared to the controls. However, no significant differences were observed in these parameters between the groups.

miRNAs Expression Levels

miRNA expression levels were assessed by qRT-PCR. Results showed a significant increase in miR-1 (+2.72 fold, p<0.0001), miR-133 (+3.041 fold, p<0.0001), miR-208a (+4.03 fold, p<0.0001), miR-206 (+1.60 fold, p=0.0026), miR-17 (+3.13 fold, p<0.0001), miR-29 (+2.48 fold, p<0.0001), miR-223 (+3.80 fold, p<0.0001), miR-326 (+1.46 fold, p=0.0021) and miR-155 (+2.58 fold, p<0.0001) expression levels in SCF patients compared to the controls (Figure 1A). Additionally, miR-15a (-0.69 fold, p=0.0004), miR-21 (-0.70 fold, p=0.0011), miR-25 (-0.60 fold, p<0.0001), miR-126 (-0.35 fold, p<0.0001), miR-17 (-0.78 fold, p=0.01), and miR-18a (-0.55 fold, p<0.0001) significantly decreased in SCF patients compared to the control group. No significant differences in miR-16 expression level were observed between the two groups (p=0.17) (Figure 1B).

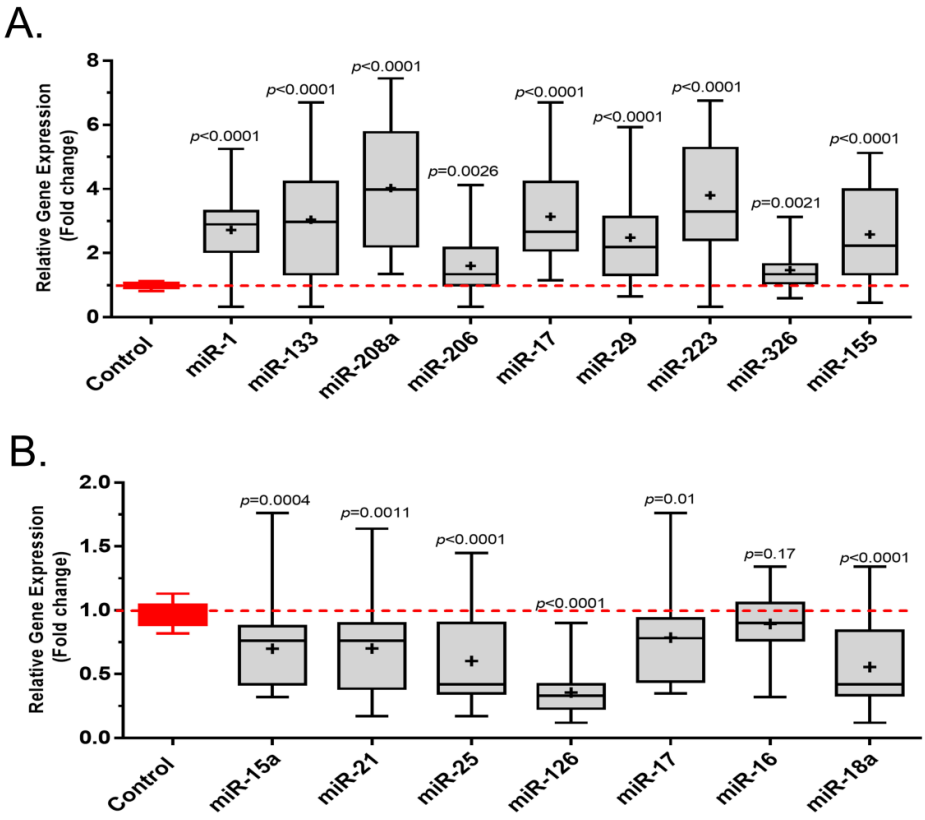

Figure 1. Quantitative RT-PCR analysis of PBMCs miRNAs in SCF and control groups. miRNAs expression in PBMCs from SCF and control groups was measured by RT-PCR. Results were normalized to RNU6B. (A) Comparative expression levels analysis of miR-1 (p<0.0001), miR-133 (p<0.0001), miR-208a (p<0.0001), miR-206 (p=0.0026), miR-17 (p<0.0001), miR-29 (p<0.0001), miR-223 (p<0.0001), miR-326 (p=0.0021), and miR-155 (p<0.0001) showed significant increase in SCF patients compared to the controls. (B) Comparative expression levels analysis of miR-15a (p=0.0004), miR-21 (p=0.0011), miR-25 (p<0.0001), miR-126 (p<0.0001), miR-17 (p=0.01), and miR-18a (p<0.0001) significantly decreased in blood samples derived from SCF patients compared to the control group. Data are expressed as mean±SD. (SCF patients, n=45; control group, n=45).

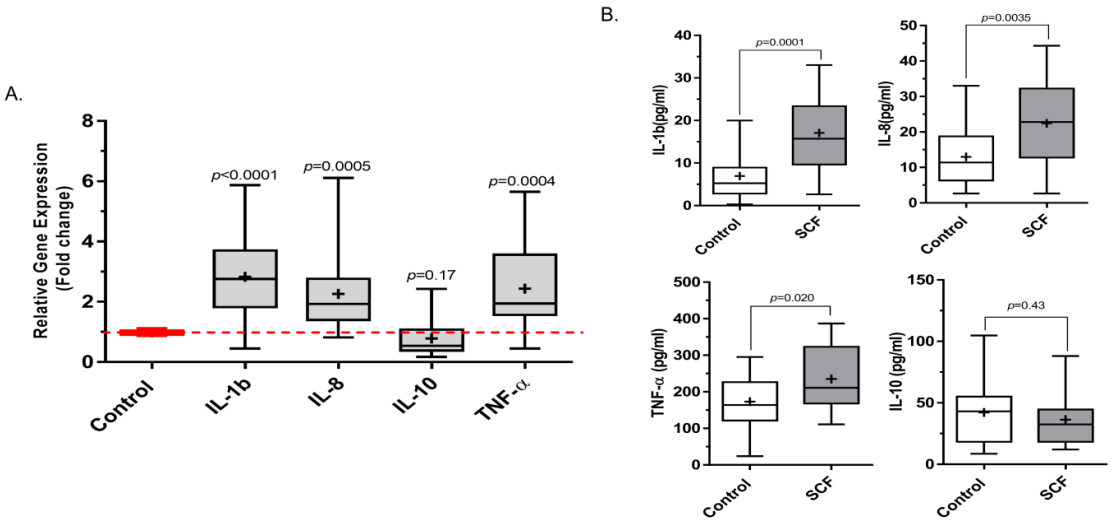

Figure 2. mRNA expression and serum concentration of cytokines. mRNA expression of cytokines in PBMCs from SCF and control groups were measured by RT-PCR. Results were normalized to β-Actin. (A) mRNA expression levels of IL-1β (p<0.0001), IL-8 (p=0.0005), and TNF-α (p=0.0004) were significantly higher in SCF patients than controls. No significant differences in IL-10 expression levels were observed between the groups (p=0.17). The serum concentration of cytokines was measured using specific cytokine ELISA detection kits. (B) Serum levels of IL-1β (p=0.0001), IL-8 (p=0.0035), and TNF-α (p=0.020) were also significantly higher in the SCF patients compared to the controls. However, no significant differences in IL-10 concentration were observed between the groups (p=0.43). Data are expressed as mean±SD. (SCF patients, n=45; control group, n=45).

Table 2. General Characteristics, risk factors, and laboratory findings in SCF patients and control group

	SCF patients (n=45)	Control group (n=45)	p value*
Age (year)	52.45±10.34	54.12±8.54	0.51
Sex(male/Female)	29/16	33/12	0.31
BMI (Kg/m^2)	27.33±3.82	29.5±4.17	0.65
Hypertension (%)	16 (35.5)	11(24.4)	0.09
Diabetes (%)	9(20)	5(11.11)	0.16
Smoker (%)	21(46.66)	23(51.11)	0.75
Total cholesterol (mg/dL)	198.5±43.56	172.87±54.3	0.02
Triglycerides (mg/dL)	145.82±33.9	132±38.85	0.87
HDL cholesterol (mg/dL)	38.65±7.33	42.56±8.58	0.65
LDL cholesterol (mg/dL)	123.21±44.65	111.2±33.57	0.21
HDL/LDL ratio	0.32±0.18	0.36±0.29	0.33
Fasting blood sugar (mg/dL)	99.55±14.81	93.76±11.43	0.69

SCF: Slow coronary flow; BMI: body mass index; LDL-C: low-density lipoprotein-cholesterol; HDL-C: high-density lipoprotein-cholesterol; FBS: fasting blood sugar. Data were presented as numbers (percentages), or means ± SD, as appropriate. p<0.05 was considered as statistically significant. *p values indicate comparison between groups (independent-sample t-test, or chi-squared test, as appropriate).

mRNA Expression and Serum Concentrations of Cytokines

To determine the effect of circulating cytokines levels in SCF patients and the control group, we investigated the cytokines expression alteration at the mRNA level on blood PBMCs. The mRNA expression levels of IL-1β (+2.83 fold, p<0.0001), IL-8 (+2.26 fold, p=0.0005) and TNF-α (+2.43 fold, p=0.0004) were significantly

higher in SCF patients than controls. No significant differences were observed in IL-10 expression levels between the two groups (0.78 fold, p=0.17) (Figure 2A). Serum levels of IL-1β (p=0.0001), IL-8 (p=0.0035), and TNF-α (p=0.020) were also significantly higher in SCF group compared to the controls. No significant differences in serum IL-10 concentration were observed between the two groups (p=0.43) (Figure 2B).

DISCUSSION

The aim of this study was to evaluate the association between inflammatory markers (cytokines) and miRNA in patients with SCF and controls. Aberrant expression or genetic deletion of miRNAs is associated with abnormal cardiac cell differentiation, disruption of heart development and cardiac dysfunction.[16] In this study, we preliminarily selected 16 miRNAs to evaluate their expression in SCF patients and controls. Our results showed an increased expression in 9 miRNAs in SCF patients compared to controls. In contrast, 6 miRNAs displayed a significantly reduced expression in SCF patients compared to control counterparts. Several studies have also demonstrated the involvement of miRNAs such as miR-1, miR-133, and miR-208 in SCF which are sufficient to prompt effective reprogramming of cardiac fibroblasts into cardiomyocytes both *in vitro* and *in vivo*.[17,18] In this context, Yang et al.[19] showed the overexpression of miR-1 in individuals with CAD. Therefore, miR-1 may be essential for pathophysiological processes in the heart and can be considered as a possible anti-arrhythmic target.[19] Additionally, miR-133 exhibits protective and anti-fibrotic properties in smooth muscles and increases in patients with coronary heart disease.[20] Even though, Fichtlscherer et al.[21] first displayed a significant reduction in most absolute and relative miRNAs levels (miR-17, miR-92a, and miR-126) together with an elevation of myocardial and inflammatory-related miRNAs (miR-133, miR-145, and miR-155) in patients with coronary heart disease. Recently, it has been reported that miR-155 can activate the ERK signaling pathway and subsequently aggravates inflammatory responses, promoting vascular reconstruction.[22] Studies also showed that plasma miR-155 levels increased in SCF patients.[23] These findings suggest that miR-155 is an important factor for SCF manifestation and may be considered as a beneficial blood biomarker reflecting the state of coronary blood flow.[23] Moreover, miR-208a is required for cardiac fibrosis development. Circulating miR-208a is also specifically expressed in the heart muscle and is involved in myosin regulation during cardiac development.[24] Previous studies reported that plasma cardiac-specific miR-208a level was significantly higher in coronary heart disease patients. These preliminary results suggest the association between miR-208a expression level and SCF.[24] Besides, miR-206 may exert an indispensable role in cardiovascular diseases. Limana et al.[25] detected increased miR-206 expression in a high mobility group box 1 (HMGB1)-simulated heart failure rat model. Another study also showed that miR-206 expression enhanced significantly in both plasma and endothelial progenitor cells (EPCs) of CAD patients.[26] Other studies also suggested that the miR-17~92 cluster revealed complex roles in endothelial cell function and angiogenesis.[27] Additionally, miR-18a-5p, miR-106a-5p, and miR-223-3p were associated with a great number of inflammatory and endothelium-related biomarkers.[28] Interestingly, Diehl, et al.[29] reported an increased miR-19-223 in acute coronary syndrome patients. In addition, bioinformatics data indicated that miR-223 regulated different cholesterol metabolism-associated genes and might also control HDL-C uptake.[29] Moreover, miR-223 was reported to be increased in familial hypercholesterolemia. Level of circulating miR-223 in expecting the severity of coronary atherosclerosis may have a relatively certain value.[30] On the other hand, miR-15a, miR-21, miR-25, miR-126, miR-17, miR-16, and miR-18a exhibited a decreased expression in our study. miR-16 is also associated with cardiovascular diseases. Since miR-16 can decrease expression of Vascular endothelial growth factor receptor 2 (VEGFR2) and Fibroblast Growth Factor Receptor 1 (FGFR1) to prevent angiogenesis, thus, its altered expression can stimulate myocardial cell hypertrophy.[31] Zheng Cao et al[32] demonstrated that higher miR-16 expression strengthened endothelial cell damage. Results of other studies also revealed that miR-16 expression increased significantly in the peripheral blood of patients with coronary heart disease compared to healthy individuals.[32] Previous research displayed that miR-126 decreased in patients with CAD. Additionally, the severity of CAD was related to the expression level of miR-126 in circulation.[33] Results of our study showed that TNF-α, IL-1β, and IL-

8 production increased significantly compared to the control group. However, no significant differences were observed in IL-10 production between SCF patients and controls. In other preclinical studies, high levels of TNF-α were reported to be involved in pathophysiology of myocarditis, cardiac allograft, and progression of congestive heart failure.[34] TNF-α is also responsible for additional ventricular remodeling, myocardial contractility decrease, endothelial cells and myocytes apoptosis enhancement, change of nitric oxide production regulating enzymes expression and function and cachexia stimulation which causes peripheral muscle dysfunction.[35] This study has some limitations that have to be pointed out. The small patient population and lack of funds did not let us draw any good conclusion. Western blot technique is also needed to confirm the association between inflammatory markers with disruption of heart development and cardiac dysfunction that will be subjected to ongoing studies.

In the present study, we discovered the potential role of some miRNAs as biomarkers for SCF diagnosis as well as CAD development monitoring in these patients. However, inflammatory markers and concurrently dynamic miRNAs provide a possible relationship among the recognized consequences of vascular inflammation and endothelial dysfunction. Thus, more investigations are still necessary to unravel the detailed essential mechanisms of decreased circulating miRNA levels in patients with heart failure and SCF.

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