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The diagnostic value of circulating microRNAs for middle-aged (40–60-year-old) coronary artery disease patients

Ali Sheikh Md Sayed,[I] Ke Xia,[I,II] Fei Li,[I] Xu Deng,[I] Umme Salma,[III] Tingbo Li,[IV] Hai Deng,[I] Dafeng Yang,[I] Zhou Haoyang,[I] TianLun Yang,[I] Jun Peng[IV]

[I] Central South University, Xiangya Hospital, Department of Cardiology, Changsha, China. [II] Harvard Medical School, Brigham and Women's Hospital, Center for Vascular Biology and Inflammation, Department of Medicine, Cardiovascular Division, Boston, U.S.A. [III] Central South University, Xiangya 3rd Hospital, Department of Gynecology and Obstetrics, Changsha, China. [IV] Central South University, School of Pharmaceutical Sciences, Department of Pharmacology, Changsha, China

OBJECTIVE: Circulating microRNAs have been recognized as promising biomarkers for various diseases. The present study aimed to explore the potential roles of circulating miR-149, miR-424 and miR-765 as non-invasive biomarkers for the diagnosis of coronary artery disease in middle-aged (40–60-year-old) patients.

METHODS: Sixty-five stable coronary artery disease patients (49–57 years old), 30 unstable coronary artery disease patients (49–58 years old), and 32 non-coronary artery disease patients (49—57 years old) who were matched for age, sex, smoking habits, hypertension and diabetes were enrolled in this study. Total RNA was isolated from plasma with TRIzol reagent. Circulating miRNA levels were measured by quantitative real-time polymerase chain reaction.

RESULTS: Circulating miR-149 levels were decreased 4.49-fold in stable coronary artery disease patients (1.18 ± 0.84) and 5.09-fold in unstable coronary artery disease patients (1.04 ± 0.65) compared with non-coronary artery disease patients (5.30 ± 2.57) ($p < 0.001$). Circulating miR-424 levels were reduced 3.6-fold in stable coronary artery disease patients (1.18 ± 0.60) and 5-fold in unstable coronary artery disease patients (0.86 ± 0.54) compared with non-coronary artery disease patients (4.35 ± 2.20) ($p < 0.001$). In contrast, circulating miR-765 levels were elevated 3.98-fold in stable coronary artery disease patients (6.09 ± 2.27) and 5.33-fold in unstable coronary artery disease patients (8.17 ± 2.77) compared with non-coronary artery disease patients (1.53 ± 0.99) ($p < 0.001$). Receiver operating characteristic curve analysis revealed that the respective areas under the curve for circulating miR-149, miR-424 and miR-765 were 0.938, 0.919 and 0.968 in stable CAD patients and 0.951, 0.960 and 0.977 in unstable coronary artery disease patients compared with non-coronary artery disease patients.

CONCLUSION: Our results suggest that circulating miR-149, miR-424 and miR-765 might be novel, non-invasive biomarkers for the diagnosis of coronary artery disease in middle-aged patients. However, future prospective trials in large patient cohorts are necessary before reaching a solid conclusion.

KEYWORDS: Circulating microRNAs; Coronary artery disease; Unstable angina; Biomarkers; Non-coronary artery disease.

◼ INTRODUCTION

Coronary artery disease (CAD) remains the leading cause of sudden cardiac death (SCD) in the world and a major cause of hospital admissions (1). The total CAD prevalence is 6.4% in US adults over 20 years of age. Among Asians over 18 years of age, the estimated incidence of CAD is 4.3%, and the prevalence of CAD will increase approximately 18% by 2030 (2). CAD is caused by the build-up of atherosclerotic plaque along the tunica intima walls of the coronary arteries of the heart, which narrows the coronary arteries and reduces blood flow to the heart. An early and correct diagnosis of CAD is important for the prevention of heart attacks and SCD. CAD is hard to diagnose without the help of the well-established invasive coronary angiogram (CAG) technique. Although ECG and ETT have been widely used, there is no specific plasma

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biomarker for the clinical diagnosis of CAD, particularly for the early diagnosis of CAD. Therefore, there is a clinical demand for specific and reliable non-invasive biomarkers for the early diagnosis of CAD.

Circulating microRNAs (miRNAs) have attracted major interest as novel biomarkers for the early diagnosis of CAD (3). miRNAs are a class of small ($\sim$22 nucleotides long), highly specific, endogenous, single-stranded, non-coding RNAs that regulate the expression of target genes by binding to the 3′ untranslated region and degrading or inhibiting the translation of mRNAs (4). It is well established that miRNAs play critical roles in physiological and pathological processes in the cardiovascular system, such as endothelial dysfunction, inflammation, apoptosis, angiogenesis, atherosclerosis, and neointimal hyperplasia or restenosis (5–8).

miRNAs in plasma or serum, called circulating miRNAs, exhibit remarkable stability and are highly resistant to plasma RNase activity due to internalization in microvesicles and the formation of protein–miRNA complexes (9–12). Therefore, the levels of individual cardiac-enriched circulating miRNAs are related to the diagnosis and prognosis of heart diseases (13). Recently, it has been demonstrated that the expression levels of circulating miRNAs are significantly altered in patients with unstable angina, acute coronary syndrome, acute myocardial infarction (AMI), heart failure, atrial fibrillation, stroke and cancer (14–21). Recently, certain circulating miRNAs, such as miR-208b, miR-499, miR-1, miR-126, miR-133, miR-1291 and miR-663b, have been recognized as novel potential biomarkers for the diagnosis of AMI (22–24). The levels of circulating miR-423-5p, miR-103, miR-142-3p, miR-30b and miR-342-3p were highly correlated with heart failure (25,26). Circulating miR-135a and miR-31 levels are up-regulated, whereas miR-378 and miR-147 levels are down-regulated in patients with stable CAD compared with healthy subjects (12). However, there have been no reports regarding circulating miRNAs as non-invasive biomarkers for the diagnosis of CAD in middle-aged patients. In this study, we detected the plasma levels of miR-149, miR-424 and miR-765 in middle-aged (40–60 years old) CAD patients to explore their potential role as biomarkers for the diagnosis of CAD in middle-aged patients.

■ MATERIALS AND METHODS

Study subjects

Thirty-two non-CAD patients, sixty-five consecutive CAD patients and thirty unstable angina (UA) patients admitted to Xiangya Hospital, Central South University, Hunan, China, from March 2012 to December 2013 were enrolled in this study. The diagnosis of CAD was confirmed by coronary angiogram. CAD was defined as at least one major coronary artery with $\geq$50% stenosis based on a modified form of the AHA/ACC classification of the coronary tree. CAD was defined as either stable or unstable according to ACC/AHA guidelines (27,28). The exclusion criteria for CAD patients were as follows: previous history of AMI; elevated cardiac troponin I (cTnI) or creatine kinase (CK-MB) levels; impaired left ventricular ejection fraction (LVEF) ($\leq$45%); congestive heart failure; cardiac arrhythmias and pacing; severe hepatic and renal dysfunction; renal replacement therapy; chronic inflammatory and malignant disease; age over 60 years; and any major operation within the previous month. The non-CAD patients were matched for age, sex, smoking habits, hypertension and diabetes, and CAD and other

diseases of interest were excluded based on the following criteria: 12-lead ECG, ETT and echocardiogram reports within normal limits; no history of stroke; no history of acute or chronic hepatic or renal disease; and no hospitalizations for at least 1 month prior to participation. The study was approved by the Local Medical Ethics Committee of Xiangya Hospital, Central South University, Hunan, China, and the protocol was performed according to the principles of the Declaration of Helsinki (2008) of the World Medical Association. All the participants provided written informed consent at the time of enrollment.

Sample collection

Peripheral 5 ml blood samples were obtained in K2-EDTA-coated tubes from the antecubital veins of CAD and non-CAD patients and were processed within 30 min of collection using two-step centrifugation. Samples were first centrifuged at $1,500 \times g$ for 15 min at 4℃. The supernatant was collected and then centrifuged again at $14,000 \times g$ for 15 min at 4℃ to obtain pure plasma. Finally, the plasma was transferred to RNase-free tubes and stored at -80℃.

RNA isolation from plasma

Total RNA was extracted using a TRIzol-based miRNA isolation protocol (Invitrogen, Carlsbad, CA, USA) (1). We added 750 µl of TRIzol reagent to 250 µl of plasma, and the resulting solution was mixed well and incubated at room temperature (RT) for 5 min prior to the addition of 200 µl of chloroform and a 3-min incubation at RT. The three phases (aqueous, inter, and organic) were obtained by centrifugation at 4℃ and 12,000 rpm for 15 min. (2). The upper aqueous phase was collected, mixed with 500 µl of 100% isopropanol and incubated at -20℃ overnight; then, the samples were centrifuged at 4℃ and 13,000 rpm for 15 min to enable RNA precipitation. RNA samples were cleaned twice with 80% ethanol (500 µl) and centrifuged again at 4℃ and 7500 rpm for 10 min (3). The supernatants were removed, and the precipitates were dried at RT for 5 min. Subsequently, the RNA samples were diluted in 30 µl of RNase-free water and incubated at 4℃ for 8 hours. Finally, the RNA concentrations of 2 µl aliquots were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA), and the RNA samples were stored at -80℃ for future use.

Detection and analysis of miRNAs by qRT-PCR

We used real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to determine the expression of miRNAs. First, 4 µL of pure RNA (OD 1.8-2.2) was reverse-transcribed (RT) to cDNA at 42℃ for 30 min using miRNA-specific reverse transcription kits (RiboBio, Guangzhou, China) according to the manufacturer's instructions with an RT-PCR system (Bio-Rad, USA). Second, 2 µL of cDNA was used as the template for qRT-PCR. Plasma miR-149, miR-424 and miR-765 expression was detected using SYBR Green miRNA qRT-PCR kits (Takara, Dalian, China) according to the manufacturer's protocol and a 7300 Real-Time PCR System (Applied Biosystems, CA, USA). A melting curve analysis was performed at the end of the PCR cycle to validate the specificity of the expected PCR product. We used miR-156a as an internal control due to its persistent and stable expression throughout all the evaluated samples. Each sample from each study subject was analyzed by PCR in triplicate. The relative expression level of each miRNA was computed using the comparative CT method, which was

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defined as $2^{-\Delta Ct}$, where ΔCt = (Ct miRNA of sample x − Ct miR-156a of sample x). The samples with qRT-PCR Ct values greater than 40 were considered not expressed. To reduce the number of false positives, we only measured miRNAs whose expression in CAD and unstable patients differed from the healthy controls by more than 2-fold on average.

Biochemical and clinical assays

Fasting blood sugar (FBS), triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (hs-CRP) were measured using an automatic analyzer (Hitach75, Tokyo, Japan). Clinical history, family history, drug history, physical examination, serial 12-lead ECG and echocardiogram reports were recorded.

Statistical analysis

Data were analyzed with SPSS software (version 20.0, SPSS, Chicago, IL) and reported as the mean ± standard deviation (SD). Differences among groups were compared using Student's t-test and one-way ANOVA; for categorical variables, Fischer's exact test or the Chi-Square (χ^2) test was used, and for non-Gaussian distributions, rank transformation and the Wilcoxon test were used. The miRNA expression data were used to generate graphs with Graph-Pad Prism version 6 for Windows (GraphPad Software,

San Diego, CA, USA) and were presented as the mean ± standard error (SEM). ROC analysis was used to assess the diagnostic accuracy of each circulating miRNA for all the groups. The area under the ROC curve (AUC) was considered a diagnostic index, and the best cut-off point was obtained based on the highest sensitivity and specificity values. All the tests were 2-sided, and differences with $p < 0.05$ were considered to be statistically significant.

■ RESULTS

Clinical participant information

We enrolled a total of 95 CAD patients, including 65 with stable CAD (49–57 years old) and 30 with unstable CAD (49–58 years old), and 32 non-CAD patients (49–57 years old) who were matched for age, sex, hypertension and diabetes. Body mass index, diabetes mellitus, systolic blood pressure, diastolic blood pressure, LVEF, FBS, TC, TG, HDL-C, LDL-C, hs-CRP, AST, ALT, creatinine, smoking habits, hypertension and treatment records were collected. Clinical and laboratory characteristics of the participants are presented in Tables 1 and 2.

The expression levels of circulating miRNAs in CAD versus non-CAD patients

We analyzed the plasma expression levels of miR-149, miR-424 and miR-765 in stable CAD, unstable CAD and non-CAD patients. The results are presented in Figure 1. The

Table 1 - Clinical data from the study subjects.

Variable	Non-CAD patients (n = 32)	CAD patients (n = 65)	UA patients (n = 30)	p_1	p_2
Age (years)	53 (49–57)	53 (49–57)	54 (49–58)	0.957	0.471
Male/Female	16/16	38/27	20/12	0.516	0.208
BMI (kg/m^2)	22 (20–23)	22 (19–25)	23 (20–27)	0.706	0.124
Active Smoker (%)	59.37	63.07	67	0.825	0.606
Hypertension (%)	56.25	70.76	73.3	0.177	0.192
Dyslipidemia (%)	37.50	41.53	43.33	0.826	0.796
DM (%)	15.63	20	23.33	0.783	0.529
SBP (mmHg)	127 (119–135)	131 (117–146)	134 (122–146)	0.330	0.018
DBP (mmHg)	79 (72–86)	80 (73–86)	82 (75–90)	0.587	0.076
LVEF (%)	66 (60–72)	63 (55–71)	62 (53–70)	0.238	0.075
Medications					
Aspirin (%)	34.3	53.84	60	0.086	0.074
β-blocker (%)	40.62	61.53	66.66	0.082	0.047
CCB (%)	31.25	38.46	46.66	0.511	0.298
ACEI/ARB (%)	28.12	35.84	40	0.502	0.423
Statin (%)	46.87	61.53	63.33	0.196	0.213
Diuretic (%)	18.75	24.61	26.66	0.612	0.550

The data are presented as the median and range. Abbreviations: UA, unstable angina; BMI, body mass index; DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVEF, left ventricular ejection fraction; CCB, calcium channel blocker; ACEI, angiotensin-converting enzyme inhibitor; and ARB, angiotensin receptor blocker. p_1-value (non-CAD patients vs. CAD patients), p_2-value (non-CAD patients vs. UA patients).

Table 2 - Laboratory data from the study subjects.

Variable	Non-CAD patients (n = 32)	CAD patients (n = 65)	UA patients (n = 30)	p_1	p_2
FBS (mmol/L)	4.9 (4.3–5.4)	5.1 (4.2–6.1)	5.2 (3.9–6.7)	0.234	0.490
TG (mmol/L)	1.5 (0.5–2.5)	1.6 (0.6–2.7)	1.8 (0.8–2.7)	0.454	0.126
TC (mmol/L)	4.4 (2.9–5.9)	4.9 (3.3–6.5)	5.3 (3.7-7)	0.177	0.038
HDL-C (mmol/L)	0.9 (0.5–1.4)	0.9 (0.4–1.4)	0.9 (0.5–1.3)	0.595	0.746
LDL-C (mmol/L)	2.7 (1.5–3.8)	2.9 (1.4–4.4)	3.2 (2.1–4.4)	0.872	0.024
hs-CRP (mg/L)	1.4 (0.5–2.7)	19.8 (9.9–29.5)	21.3 (15–27.5)	< 0.001	< 0.001
AST (U/L)	15.3 (6.9–24)	17.7 (8.3–27.1)	19.8 (12.6–27)	0.294	0.019
ALT (U/L)	17.1 (9.1–25)	19.1 (10.7–27.4)	21.5 (13.4–29.6)	0.244	0.042
Cr (umol/L)	71.5 (52.4–90)	74.8 (53.4–96.1)	79.8 (58.6–101.1)	0.602	0.049

The data are presented as the median and range. Abbreviations: FBS, fasting blood sugar; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; and Cr, creatinine. p_1-value (CAD patients vs. non-CAD patients), p_2-value (UA patients vs. non-CAD patients).

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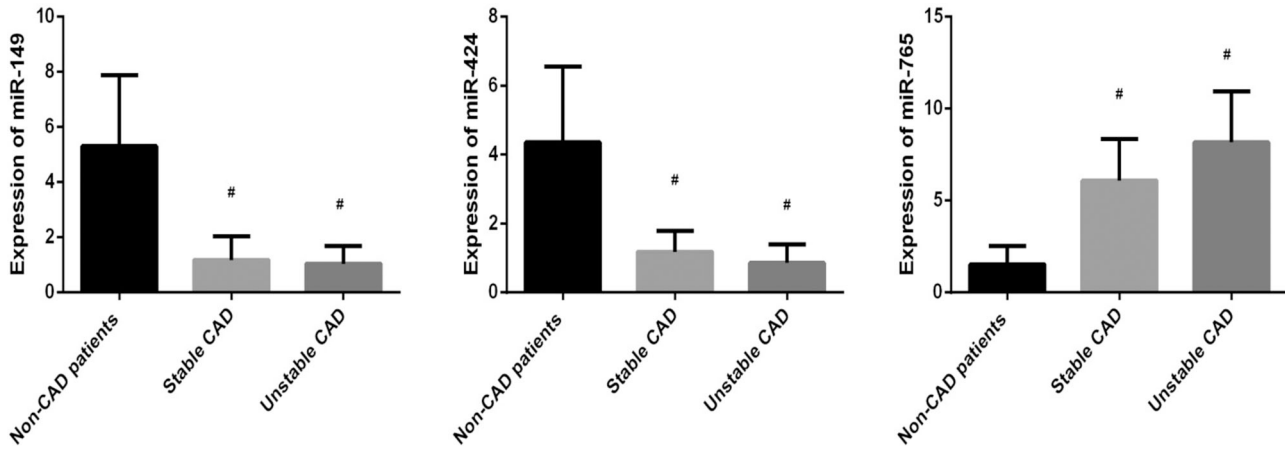Md Sayed AS et al.

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Figure 1 - Plasma expression levels of miR-149, miR-424 and miR-765 in non-CAD patients, stable CAD patients and unstable CAD patients.

levels of plasma miR-149 in stable CAD and unstable CAD patients were 4.49-fold (1.18 ± 0.84) and 5.09-fold (1.04 ± 0.65) lower, respectively, than those in non-CAD patients (5.30 ± 2.57) ($p < 0.001$). Circulating miR-424 levels were down-regulated 3.6-fold (1.18 ± 0.60) in stable CAD patients and 5-fold (0.86 ± 0.54) in unstable CAD patients compared with those in non-CAD patients (4.35 ± 2.20) ($p < 0.001$). In contrast, the plasma concentrations of miR-765 were elevated 3.98-fold (6.09 ± 2.27) in stable CAD patients and 5.33-fold (8.17 ± 2.77) in unstable CAD patients compared with those in non-CAD patients (1.53 ± 0.99). The plasma levels of miR-149 and miR-424 were not significantly different between stable and unstable CAD patients, whereas the plasma levels of miR-765 were significantly different between stable and unstable CAD patients ($p < 0.001$).

Diagnostic role of plasma miRNAs in middle-aged CAD patients

To examine the potential of miRNAs (miR-149, miR-424, and miR-765) as biomarkers for middle-aged CAD patients, ROC analysis was performed on all 127 subjects. The ROC curves of plasma miR-149 revealed a strong discrimination between non-CAD patients, stable CAD patients and unstable CAD patients, with AUCs of 0.938, 0.951 and 0.539, respectively (Figure 2A, 2B, and 2C). The ROC curves of plasma miR-424 revealed significant differences between non-CAD patients, CAD patients and unstable CAD patients, with AUCs of 0.919,

0.960 and 0.682, respectively (Figure 2D, 2E, and 2F). Furthermore, the ROC curves of plasma miR-765 also accurately differentiated stable and unstable CAD patients from non-CAD patients, with AUCs of 0.968, 0.977 and 0.760, respectively (Figure 2G, 2H, and 2I). These results indicated that plasma miR-149, miR-424 and miR-765 might be valuable biomarkers for middle-aged CAD patients. The AUCs, maximum cut-off points, sensitivities, specificities, 95% CIs (confidence intervals) and p-values of the circulatory miRNAs (miR-149, miR-424, and miR-765) are briefly summarized in Table 3.

■ DISCUSSION

CAD is the most important cause of heart attacks and resulted in 3.6 million deaths in the year of 1990 in developing countries; this number will approximately increase to 7.8 million deaths by the year 2020 (29). The main cause of CAD is atherosclerosis, which is a hyperlipidemia-induced chronic inflammatory disease of the arterial wall that occurs predominantly at sites of disturbed laminar blood flow due to endothelial dysfunction. Emerging evidence suggests that alterations in flow conditions regulate the expression of miRNAs in endothelial cells both in vitro and in vivo (30). The administration of miR-126-5p rescued EC proliferation at predilection sites and limited atherosclerosis by suppressing the Notch1 inhibitor delta-like 1 homolog (Dlk1), introducing a potential therapeutic approach (31). miR-33 has

Table 3 - Receiver operator characteristic curve (ROC) analysis of miRNA ratios for predicting coronary artery disease.

miRNA	AUC	Cut-off point	Sensitivity	Specificity	95% CI	*p*-value
miR-149						
Non-CAD vs. CAD	0.938	1.67	71.8%	95.3%	0.894 to 0.983	< 0.0001
Non-CAD vs. UA	0.951	1.71	75%	96.6%	0.905 to 0.997	< 0.0001
CAD vs. UA	0.539	1.15	75%	40%	0.415 to 0.663	0.540
miR-424						
Non-CAD vs. CAD	0.919	1.61	68.7%	92.3%	0.863 to 0.975	< 0.0001
Non-CAD vs. UA	0.960	1.77	84.3%	93.3%	0.920 to 1.000	< 0.0001
CAD vs. UA	0.682	1.35	89.2%	46.6%	0.559 to 0.805	0.005
miR-765						
Non-CAD vs. CAD	0.968	1.75	81.5%	93.7%	0.939 to 996	< 0.0001
Non-CAD vs. UA	0.977	1.80	83.3%	96.8%	0.949 to 1.005	< 0.0001
CAD vs. UA	0.760	1.57	66.5%	90.7%	0.635 to 0.885	< 0.0001

AUC, area under the curve; CAD, coronary artery disease; Non-CAD, non-coronary artery disease; UA, unstable angina (unstable coronary artery disease); 95% CI, confidence interval.
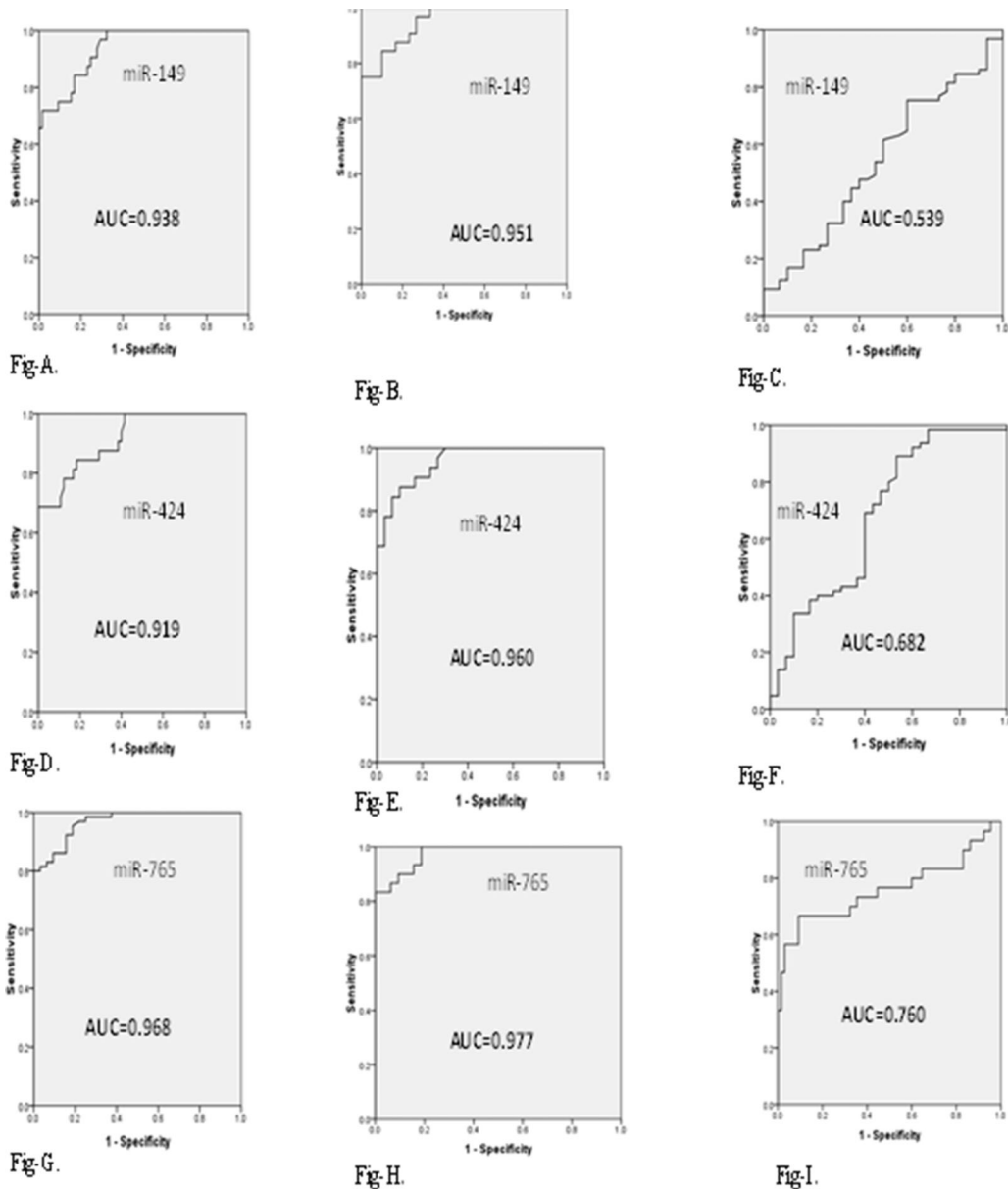
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Figure 2 - Diagnostic accuracy of circulating miR-149, miR-424 and miR-765 were analyzed by ROC curve. **A)** ROC curve of miR-149, non-CAD patients vs. CAD. **B)** ROC curve of miR-149, non-CAD patients vs. unstable CAD patients. **C)** ROC curve of miR-149, CAD vs. unstable CAD patients. **D)** ROC curve of miR-424, non-CAD patients vs. CAD. **E)** ROC curve of miR-424, non-CAD patients vs. unstable CAD patients. **F)** ROC curve of miR-424, CAD vs. unstable CAD patients. **G)** ROC curve of miR-765, non-CAD patients vs. CAD. **H)** ROC curve of miR-765, non-CAD patients vs. unstable CAD patients. **I)** ROC curve of miR-765, CAD vs. unstable CAD patients.

been discovered as a key regulator of the initiation and progression of atherosclerosis by controlling lipid metabolism, the inflammatory response and cell cycle progression and proliferation (32). miR-1 prevents high-cholesterol-induced endothelial dysfunction through myosin light chain kinase (MLCK) expression and extracellular signal-regulated kinase (ERK) phosphorylation (33). Monocytes migrate from the circulation into the intima by binding to activated endothelial cells and later differentiate into macrophages. miR-424 promotes monocyte/macrophage differentiation and is intimately involved in the initiation and progression of atherosclerotic lesions and the development of associated complications. Ezetimibe (a lipid-lowering agent) decreased the expression of miR-424 by 75% and decreased atherosclerotic lesions (34).

In addition, several miRNAs are directly and indirectly involved in the pathophysiological mechanisms of atherosclerosis, such as miR-19a, miR-21, miR-23b, miR-92a, miR-143, miR-145, miR-155, miR-663 and miR-133a (30,35). Although enormous progress has been made in the diagnosis, treatment and prognosis of CAD, there remains a clinical need for a novel diagnostic biomarker and new therapeutic interventions to decrease the incidence of CAD. Biomarkers play an essential role in the diagnosis of CAD. However, many of these biomarkers have shortcomings, such as reduced sensitivity, low specificity or unsuitability for early diagnosis. However, several recent studies have suggested that circulating miRNAs are excellent promising biomarkers for the early diagnosis of AMI (18,23–25,36).

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Circulating levels of miR-133a, miR-208a, miR-1, miR-122, miR-133b, miR-337-5p, miR-433, and miR-485-3p were significantly up-regulated, whereas miR-126, miR-17, miR-92a, miR-145, miR-155, and miR-199a levels were markedly down-regulated in CAD (14,37).

Circulating miRNA levels in middle-aged CAD patients have not yet been investigated. We specifically focused on middle-aged people for the following reasons: one, they are the most active contributors to the development of society; and two, although the invasive CAG procedure is the standard method for diagnosing CAD, it has many complications. The present work demonstrated that circulating miRNAs might be clinically feasible biomarkers for diagnosing middle-aged CAD patients. We discovered that plasma miR-149 and miR-424 levels were significantly down-regulated, whereas plasma miR-765 levels were up-regulated in stable and unstable CAD patients compared with non-CAD patients. In addition, we measured the diagnostic value of plasma miR-149, miR-424 and miR-765 levels using ROC curves. The AUC values for miR-149, miR-424 and miR-765 were significantly higher in stable CAD patients (0.938, 0.919 and 0.968, respectively) and unstable CAD patients (0.951, 0.960 and 0.977, respectively) compared with non-CAD patients. These results strongly indicated that plasma miR-149, miR-424 and miR-765 might be valuable novel non-invasive biomarkers for the early diagnosis of middle-aged CAD patients.

Recent studies have revealed that 5,10-methylenetetrahydrofolate reductase (MTHFR) plays a critical role in homocysteine metabolism. The abnormal function of MTHFR increases plasma homocysteine levels to enhance endothelial dysfunction and atherosclerosis. The human MTHFR rs4846049 (T-allele) polymorphism was significantly associated with an increased risk of CAD and dyslipidemia via modification of the binding of miR-149 in a Chinese Han population (38). Furthermore, the *miR-149*T > C polymorphism was significantly associated with ischemic stroke and silent brain infarction (SBI) risk in a Korean population (39). TNF-α plays an essential role in the initiation and progression of endothelial dysfunction. The transfection of miR-149 mimics counteracted the TNF-α-induced expression of metalloproteinase-9 (MMP-9), inducible nitric oxide synthase (iNOS), and interleukin-6 (IL-6) and regulated TNF-α-induced endothelial dysfunction (40). miR-149 expression was significantly down-regulated in infarcted mouse hearts three days after MI and in cardiac tissue from the border zone of the infarcted region in patients receiving a cardiac transplant (41).

In our study, circulating miR-424 levels were significantly decreased in CAD patients, which is consistent with studies from Fichtlscherer et al (14). Moreover, plasma miR-424 levels were significantly down-regulated in acute ischemic stroke patients as well as in mouse plasma and brain tissue 4 hours after ischemia. In addition, overexpressing miR-424 mimics decreased the ischemic brain injury by suppressing microglia activation via the translational depression of key activators of the G1/S transition, including CDC25A, CCND1, and CDK6 (42). miR-424 levels were also significantly decreased in pulmonary arterial hypertension (PAH) due to the dysregulation of the apelin (APLN) and fibroblast growth factor 2 (FGF2) signaling pathways (43). The specific target of miR-424 in cardiac cells is currently unknown and awaits elucidation in future studies.

Redell JB et al. (44) reported that plasma miR-765 levels were markedly increased in traumatic brain injury (TBI) patients compared with healthy volunteers. ROC curve analysis indicated that miR-765 was a good biomarker, especially for severe TBI. However, the exact mechanisms for the altered expression of miR-765 in CAD remain unknown. Using target scan (www.targetscan.org), hsa-miR-765 was predicted to potentially be involved in CAD by regulating several important target genes, such as HIF3A (hypoxia inducible factor-3, α subunit), NADPH oxidase, low density lipoprotein receptor-related protein 4 (LRP4), and low density lipoprotein receptor-related protein 6 (LRP6).

To ensure that the study populations were comparable, subjects with similar clinical features, such as age, gender, TC, TG, HDL-C, LDL-C, systolic blood pressure, diastolic blood pressure, AST, ALT, creatinine, LVEF, diabetes mellitus, smoking, hypertension and medication history, were carefully considered. Statistical analyses revealed that these factors did not affect circulating miR-149, miR-424 and miR-765 levels. Importantly, three plasma miRNAs (miR-149, miR-424 and miR-765) displayed a direct relationship in stroke patients, but we excluded stroke patients in our study to minimize possible bias.

To decrease potential errors resulting from qRT-PCR assays, we evaluated three potential internal controls (U6, miR-cel-39, and miR-156a) in a pre-analysis (n = 20). We ultimately chose miR-156a for standard normalization because it was more stable and reliable (36). We analyzed each sample in triplicate, and miRNAs with Ct values ≥40 were not included in our study, which assured that our results were constant and reproducible.

To the best of our knowledge, the present study is the first to demonstrate that plasma miR-149, miR-424 and miR-765 might be non-invasive novel biomarkers for middle-aged CAD patients. However, there are several limitations that need to be acknowledged and addressed regarding the present study. First, this work was performed in a single center with a small cohort. Therefore, a multi-center study with a larger cohort is necessary to verify the findings of this present study. Second, the specificity of the changes in the three measured miRNAs in middle-aged CAD patients remains unclear because we did not measure these miRNAs in other age groups of CAD patients or in other clinical settings. Third, we did not include healthy subjects as normal controls.

In conclusion, our findings suggest that circulating miR-149, miR-424 and miR-765 might be non-invasive biomarkers for the diagnosis of CAD in middle-aged patients. However, the sensitivity and specificity of these miRNAs need to be further examined in a larger clinical cohort.

■ AUTHOR CONTRIBUTIONS

Sayed AS and Xia K designed the research, performed the experiments and wrote the manuscript. Deng X, Deng H, Yangda F, Li F, Haoyang Z, Li T, and Salma U collected patient samples and assembled the data. Yang T and Peng J supervised the data analysis and revised the manuscript. The work presented in this paper was performed in collaboration with all the authors.

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