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Circulating microRNAs as biomarkers for severe coronary artery disease

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Abstract

Coronary artery disease (CAD) is the second leading cause of death after stroke in China. Percutaneous coronary intervention (PCI) significantly improves the prognosis of CAD patients. This study aimed to evaluate the diagnostic value of circulating microRNAs (miRNAs) in patients with severe CAD requiring PCI. The plasma miRNA profiles were determined using miRNA microarray. The relative expression levels of differentially expressed miRNA were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Nine miRNAs (ebv-miR-BART12, ebv-miR-BART16, let-7i-5p, miR-130a-3p, miR-26a-5p, miR-3149, miR-3152–3p, miR-32-3p, and miR-149-3p) were differentially expressed between severe CAD and control groups. Four miRNAs (let-7i-5p, miR-32-3p, miR-3149, and miR-26a-5p) validated by qRT-PCR showed good diagnostic accuracy, with the area under the receiver operating characteristic curves (AUCs) of 0.634 (95% confidence interval [CI] 0.528–0.739), 0.745 (95%CI 0.649–0.84), 0.795 (95%CI 0.709–0.88), and 0.818 (95%CI 0.739–0.897), respectively. Furthermore, the combination of these 4 miRNAs exhibited better diagnostic performance compared with any individual miRNA, with an AUC of 0.837 (95%CI 0.763–0.911). These data indicate that plasma let-7i-5p, miR-32–3p, miR-3149, and miR-26a-5p have promising diagnostic value for severe CAD.

Abbreviations: AF = atrial fibrillation AMI = acute myocardial infarction, AUC = area under the ROC curve, CAD = coronary artery disease, CI = confidence interval, miRNA = microRNA, PCI = percutaneous coronary intervention, qRT-PCR = quantitative reverse transcription polymerase chain reaction, ROC = receiver operating characteristic, VSMC = vascular smooth muscle cell.

Keywords: coronary artery disease, diagnosis, microRNA, percutaneous coronary intervention

1. Introduction

Coronary artery disease (CAD) is the leading cause of death worldwide, accounting for 16% of all deaths, followed by stroke (11%).[1] The death rate of CAD has continued to increase in the last decade, partly as a result of obesity and lifestyle changes.[2,3] In China, about 15% of the deaths are caused by CAD, ranking the second leading cause of death behind stroke (21%).[4] Continued high levels of exposure to metabolic and behavioral risk factors could contribute to a situation in the future whereby the incidence of CAD will be still increasing.[5] Approximately 7% of CAD patients are treated with percutaneous coronary intervention (PCI).[6] Although PCI significantly improves the prognosis of CAD patients, 0.23% of the patients die after receiving PCI.[6] Furthermore, some patients with normal or nonspecific electrocardiograms need PCI when they are diagnosed with CAD.[7] Therefore, identification of biomarkers for early diagnosis of patients with severe CAD is important to improve their prognosis.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules with a length of ~22 nucleotides. MiRNAs play pivotal roles in various biological processes through negatively regulating expression of their target genes at the posttranscriptional level.[8,9] Dysregulation of miRNAs contributes to the occurrence, development, and progression of human diseases, including cancer and CAD.[9–14] In addition, miRNAs are also stably present in the extracellular space, such as blood, urine, cerebrospinal fluid, tears, and saliva[15,16]. Extracellular miRNAs can be internalized by the recipient cells and serve as intercellular messengers to affect many physiological processes.[16] Many studies have revealed that circulation miRNAs are potential biomarkers for the diagnosis and prognosis of diseases.[12,17–19] For example, miR-3149 may be a novel biomarker for acute myocardial infarction (AMI).[19] In the present study, we investigated the potential of plasma miRNAs as biomarkers for patients with severe CAD requiring PCI.

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2. Materials and methods

2.1. Patients

The study population was enrolled between December 2018 and July 2019. CAD patients who underwent PCI with stent implantation for the first time in Taizhou Central Hospital were selected. All CAD patients were confirmed by angiographic evidence of >70% stenosis of at least 1 main coronary artery. The exclusion criteria were as follows:

(1) patients without successful PCI;
(2) history of AMI or prior PCI;
(3) patients with valvular heart disease, AMI, cardiomyopathy, cancer, active infection diseases, immune diseases, decompensated or severe liver diseases, and renal dysfunction.

A total of 40 patients with severe CAD were included in the study. Furthermore, controls were selected from patients with suspected cardiac chest pain who underwent diagnostic coronary angiography in Taizhou Central Hospital. Finally, 69 individuals with normal coronary angiogram and no atherosclerotic vascular disease were considered as non-CAD patients and included in the control group. Furthermore, 4 patients with severe CAD and 4 healthy controls were further enrolled to assess the unique miRNA expression profile. Venous blood samples were collected before coronary angiography. The study was approved by the Ethics Committee of Taizhou Central Hospital (2018–025). The written informed consent was obtained from all participants before participation.

2.2. RNA isolation

Five milliliters of venous blood ethylenediaminetetraacetic acid (EDTA) were collected into EDTA containing tubes and then centrifuged at 3000 revolutions per minute (rpm) for 15 minutes at room temperature to obtain plasma within 2 hours. Plasma was further centrifuged again to remove blood cells and platelets and then was stored at –80°C. RNAs were isolated from plasma samples using the mirVana PARIS Kit (Ambion, Thermo Fisher Scientific, Waltham, MA). Briefly, 500 μL of plasma was mixed with 500 μL of denaturing solution according to the manufacturer's instructions. The homogenate was then incubated at room temperature for 5 minutes. Furthermore, 5 μL of 5 nM synthetic Cel-miR-39–3p was added to each plasma sample during processing to normalize sample-to-sample variation in RNA isolation. Subsequently, the RNA was extracted according to the manufacturer's protocols and resuspended in a total volume of 50 μL RNase-free water. The quantity and quality of RNAs were measured by NanoDrop ND-2000 Spectrophotometer (Thermo Scientific Wilmington, DE).

2.3. Microarray analysis

The miRNA expression profiling was performed using the Agilent human miRNA array (Agilent Technologies, CA) among 4 CAD patients receiving PCI and 4 healthy controls. The sample preparation, microarray hybridization, and washing were carried out according to the manufacturer's standard instructions. The arrays were scanned with the Agilent Scanner G2505C (Agilent Technologies, CA). The raw data were analyzed using GeneSpring GX software (version 13.1, Agilent Technologies, CA), including data summarization, normalization, and quality control. Differential expression of miRNAs was considered as significant when a relative fold change >2 or < –2 and a *P* value was less than .05.

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The cDNA was synthesized using Mir-X miRNA First-Strand Synthesis kit (Takara, Dalian, China) according to the manufacturer's protocols. The expression levels of miRNA were measured using Mir-X miRNA qRT-PCR Kit (Takara, Dalian, China) on an ABI 7900 real-time PCR system (Applied Biosystems, CA). Exogenous cel-miR-39–3p was used as a control. The relative expression levels of miRNAs in plasma were calculated using the equation $2^{-\Delta\Delta Ct}$.

2.5. Statistical analyses

All statistical analyses were carried out using SPSS v25.0 software (SPSS, Inc., Chicago, IL). The Student *t* test and Wilcoxon rank-sum test were used for comparison of quantitative variables between CAD and control groups, whereas Fisher exact test was used for comparison of categorical variables between groups. Diagnostic performance of plasma miRNAs was evaluated using receiver operating characteristic (ROC) curve analysis. Logistic regression was employed to evaluate the joint power of miRNAs at distinguish patients with severe CAD from control. The area under the ROC curve (AUC) was used to assess the diagnostic performance of miRNAs. A *P* value <.05 (2-sided) was considered as statistically significant.

3. Results

3.1. Clinical characteristics of patients with severe CAD and controls

The clinical characteristics of 40 patients with severe CAD and 69 controls are summarized in Table 1. Patients with severe CAD had higher levels of blood pressure and total cholesterol compared with controls (*P* <.05). There was no significant difference in age, sex, heart rate, smoking history, alcohol consumption, diabetes history, fasting plasma glucose, triglyceride, low-density lipoprotein-cholesterol, and high-density lipoprotein-cholesterol (*P* >.05).

Table 1

Clinical characteristics in CAD patients and control subjects.

Characteristics	Control (n=69)	CAD (*n*=40)	*P* value
Age, yr	56.2±7.6	55.0±6.5	.370
Male, n (%)	30 (75.0%)	43 (62.3)	.208
Systolic blood pressure, mm Hg	134±18	124±17	.002
Diastolic blood pressure, mm Hg	73±13	80±11	.031
Heart rate, beats per minute	79±7	77±8	.227
Fasting plasma glucose, mmol/L	6.0±1.5	5.7±2.0	.425
Hypertension, n (%)	29 (72.5%)	20 (29.0)	<.001
Diabetes mellitus, n (%)	7 (17.5%)	4 (5.8)	.095
Smoker, n (%)	19 (47.5)	20 (31.3)	.102
Drinker, n (%)	10 (25.0)	13 (19.7)	.628
Total cholesterol, mmol/L	5.0±1.0	4.5±0.9	.017
Triglycerides, mmol/L	1.9±1.5	1.4±0.9	.054
HDL-C, mmol/L	1.4±0.3	1.6±0.3	.070
LDL-C, mmol/L	2.9±0.8	2.8±0.7	.118

CAD = coronary artery disease, HDL-C = high-density lipoprotein-cholesterol, LDL-C = low-density lipoprotein-cholesterol.

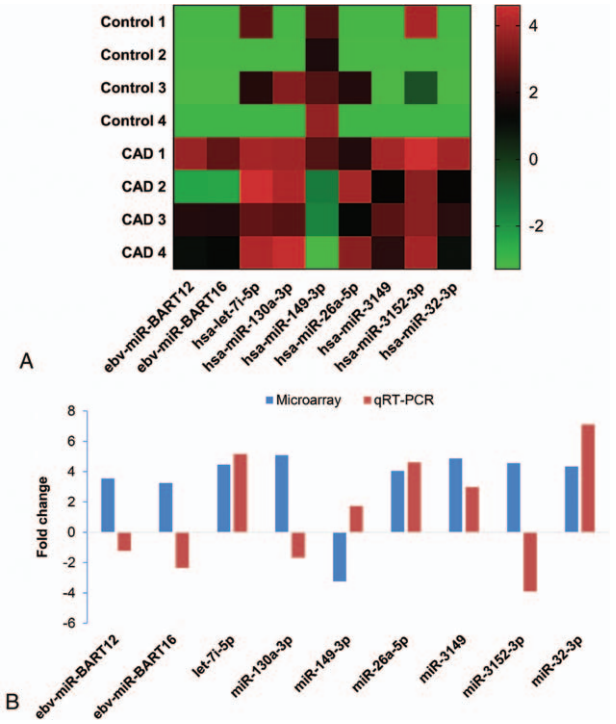

Figure 1. MiRNA expression profiles. A, the heat map indicates miRNA profiles. Each column represents an miRNA. Green and red represent low and high expressions, respectively. B, differentially expressed miRNAs were validated by qRT-PCR.

3.2. Plasma miRNA profiling of patients with severe CAD

Microarray analysis revealed that 9 miRNAs were differentially expressed between CAD and control groups. Eight miRNAs (ebv-miR-BART12, ebv-miR-BART16, let-7i-5p, miR-130a-3p, miR-26a-5p, miR-3149, miR-3152–3p, miR-32-3p) were significantly upregulated, whereas miR-149-3p was significantly downregulated in CAD plasma samples compared with the control group (Fig. 1A). We further used qRT-PCR to validate differentially expressed miRNAs. Four miRNAs (let-7i-5p, miR-32-3p, miR-3149, and miR-26a-5p) showed consistent results between qRT-PCR and microarray (Fig. 1B). Therefore, we further assessed the expression levels of these 4 miRNAs in plasma samples from 40 severe CAD cases and 69 controls with qRT-PCR. As shown in Figure 2, circulating expression levels of let-7i-5p, miR-32-3p, miR-3149, and miR-26a-5p in CAD were higher than those in controls ($P < .05$).

3.3. Diagnostic value of plasma miRNAs in severe CAD

To investigate the possibility of these miRNAs as new and potential biomarkers for CAD requiring PCI, ROC curves were constructed. As shown in Figure 3, the AUC values of let-7i-5p, miR-32-3p, miR-3149, and miR-26a-5p were 0.634 (95% confidence interval [CI] 0.528–0.739), 0.745 (95%CI 0.649–0.84), 0.795 (95%CI 0.709–0.88), and 0.818 (95%CI 0.739–0.897), respectively. These results revealed that let-7i-5p, miR-32–3p, miR-3149, and miR-26a-5p had significant sensitivity and specificity for CAD. Furthermore, the combination of these 4 miRNAs exhibited better diagnostic performance compared with any individual miRNA, with an AUC of 0.837 (95%CI 0.763–0.911).

4. Discussion

Although CAD is one of the most common deadly heart diseases, it is treatable when detected early. The serious burden of CAD, as well as high rates of major adverse events including acute myocardial infarction and death, underlines the need to identify and provide treatment to CAD patients at high risk for these major adverse events. Except for disease-modifying guideline-directed medical therapy, a considerable proportion of CAD patients need further coronary revascularization to improve quality of life and reduce adverse clinical events. However, some patients with severe CAD did not receive timely effective treatment due to atypical clinical symptoms. In the present study, we herein investigate the expression of circulating miRNAs in patients with severe CAD. The panel of 4 plasma miRNAs (let-7i-5p, miR-32–3p, miR-3149, and miR-26a-5p) can discriminate severe CAD cases from controls with a high AUC of 0.837.

Circulative miRNAs present as a source of novel non-invasive diagnostic and prognostic biomarkers for disease, such as cardiovascular diseases, neurological disorders, and cancer.[12,16–19] Although ROC analysis showed that significant differentially expressed miRNAs were able to distinguish patients with severe CAD from controls, the performance of these individual plasma miRNAs except for miR-26a-5p was not yet satisfactory (AUC = 0.634–0.795). Based on the logistic regression model, combination of these 4 miRNAs as a panel exhibited a high diagnostic efficiency for severe CAD (AUC = 0.837). The plasma miRNA panel significantly enhances the diagnostic efficiency of severe CAD compared with that of any single miRNA, which could provide a strategy using plasma miRNA biomarkers to screen patients with severe CAD. In fact, many studies have explored the diagnostic value of circulating miRNA panels in MI.[19–21] However, there are only a few reports investigating the role of miRNAs as potential non-invasive biomarkers for evaluating the severity of CAD patients without AMI.[22] Further large-scale studies are necessary to validate their potential applicability in determination of CAD severity.

Let-7 is an evolutionarily conserved family of miRNAs that consists of 13 members in human located in Chromosome 9.[23] Let-7 family miRNAs are highly expressed in somatic cells and play important roles in development, cell differentiation, and metabolism, as well as human diseases.[13,23,24] For example, let-7i suppresses progesterone-induced functional recovery after ischemic stroke through inhibiting expression of progesterone receptor membrane component 1 and brain-derived neurotrophic factor in glia.[25] A recent study by Hu et al[24] showed that let-7i-5p was a strong suppressor of cardiomyocytes proliferation and the suppression of let-7i-5p promoted cardiac repair and improved heart function after myocardial infarction. Furthermore, let-7i is downregulated in dilated cardiomyopathy and low expression of let-7i is associated with poor clinical outcomes in patients with dilated cardiomyopathy.[26] However, in the present study, we found an elevated level of circulating let-7i-5p in patients with severe CAD. Atorvastatin treatment can markedly upregulate let-7i expression of monocytes of CAD patients.[27] Therefore, patients receiving statin treatment may have a high level of circulating let-7i-5p. Furthermore, many patients with severe CAD received statin treatment. Taken together, this could partly explain high level of circulating let-7i-5p in patients with severe CAD. Since sample size was small, further studies are warranted to verify these findings.

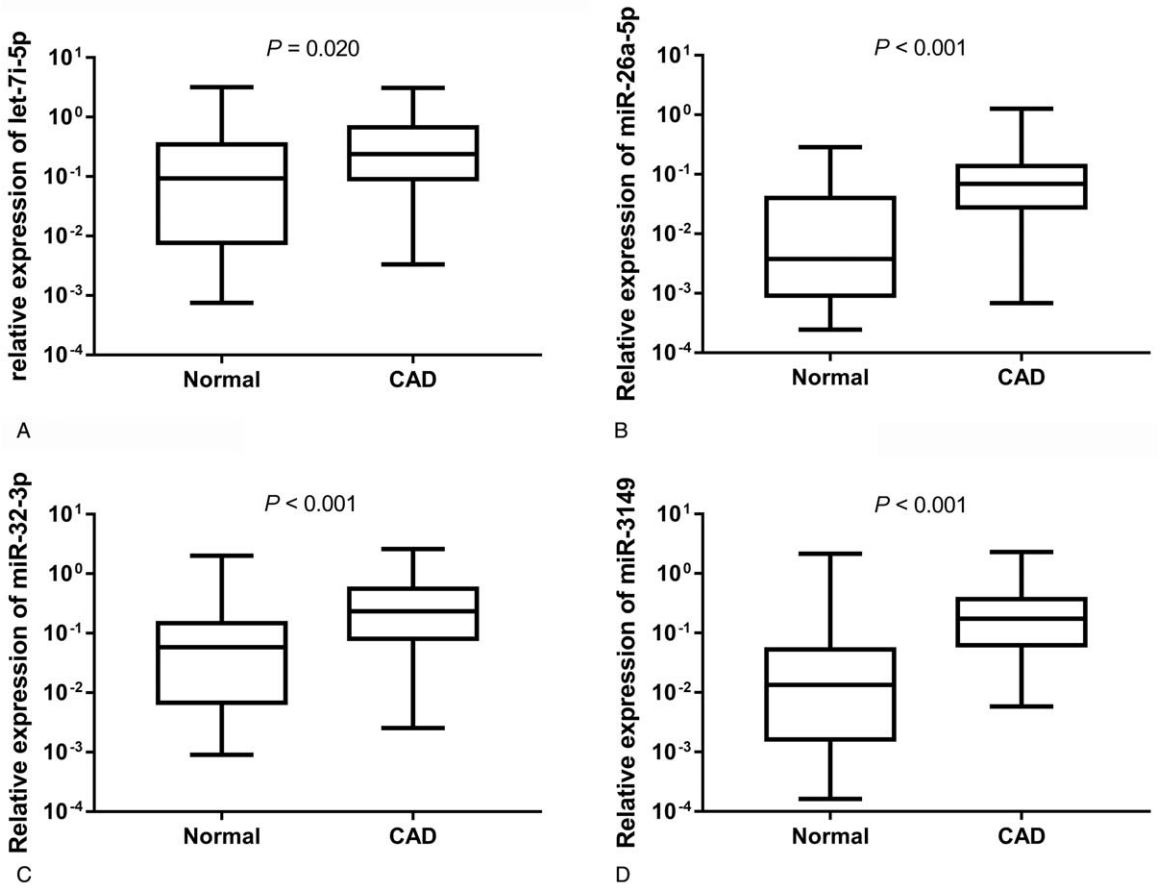

Figure 2. Plasma expression levels of miRNAs in patients with severe coronary artery disease and controls. The expression levels of let-7i-5p (A), miR-26a-5p (B), miR-32-3p (C), and miR-3149 (D) of patients with severe coronary artery disease were significantly higher than those of controls.

MiR-3149, located at 8q21.13, is highly expressed in heart.[21] A study by Xu et al[28] identified that plasma miR-3149 level was significantly increased from the early to end stages of atrial fibrillation (AF), which may reflect the severity and progression of AF. Since plasma levels of miR-3149 in peripheral blood of AF patients were significantly higher than those in coronary sinus blood of patients and controls, elevated plasma miR-3149 level was mainly originated from extracardiac tissues or cells other than cardiac cells. Li et al[19,21] also found that circulating miR-3149 level was increased in AMI, which was mainly originated from circulating endothelial cells. Therefore, elevated plasma miR-3149 levels in patients with severe CAD may also be originated from circulating endothelial cells. Given that circulating miR-3149 level is increased in AF, AMI, and severe CAD, other heart disease might have elevated plasma miR-3149 level. MiR-3149 might be a novel biomarker for heart diseases, such as AF and severe CAD.

MiR-32 could act as a positive modulator of vascular smooth muscle cell (VSMC) calcification. Liu et al[29] found that miR-32 promoted VSMC calcification by enhancing expression of alkaline phosphatase, bone morphogenetic protein-2, runt-related transcription factor-2, osteopontin, and the bone-specific phosphoprotein matrix GLA protein through AKT/phosphatase and tensin homolog pathway. In addition, CAD patients with coronary artery calcification had higher plasma levels of miR-32 and alkaline phosphatase. Therefore, miR-32 might be a potential diagnostic biomarker for coronary artery calcification. A recent study by Shen et al[30] found that overexpression of miR-32–5p induced by high glucose stimulation enhanced the apoptosis of cardiac fibroblasts, indicating that miR-32–5p may be involve in the cardiac fibrosis. Hou et al[31] identified a circulating miRNA profile containing miR-32–5p associated with blood stasis syndrome of CAD patients. In this study, we observed elevated plasma miR-32–3p levels in patients with severe CAD, implying that miR-32–3p may participate in the progression of CAD. Taken together, miR-32 may play an important role in the pathophysiological process of CAD.

It has been demonstrated that dysregulation of miR-26a-5p is associated with a variety of human diseases, such as cancer[14] and cardiovascular diseases.[32] For example, Peng et al[33] reported that miR-26a was downregulated in the peripheral blood of patients with abdominal aortic aneurysm, whereas enhanced miR-26a expression can protect VSMCs of abdominal aorta against H_2O_2-induced ROS accumulation and apoptosis by reactivating the phosphatase and tensin homolog /AKT/mammalian target of rapamycin pathway, indicated that miR-26a may be a potential therapeutic target for abdominal aortic aneurysm. However, a study by Tan et al[34] showed that miR-26a inhibited jugular vein VSMCs proliferation and vein graft neointimal hyperplasia through the mitogen-activated protein kinase 6 pathway. A recent study by Zheng et al[35] found that miR-26a-5p regulated the autophagy in cardiac fibroblasts and cardiac

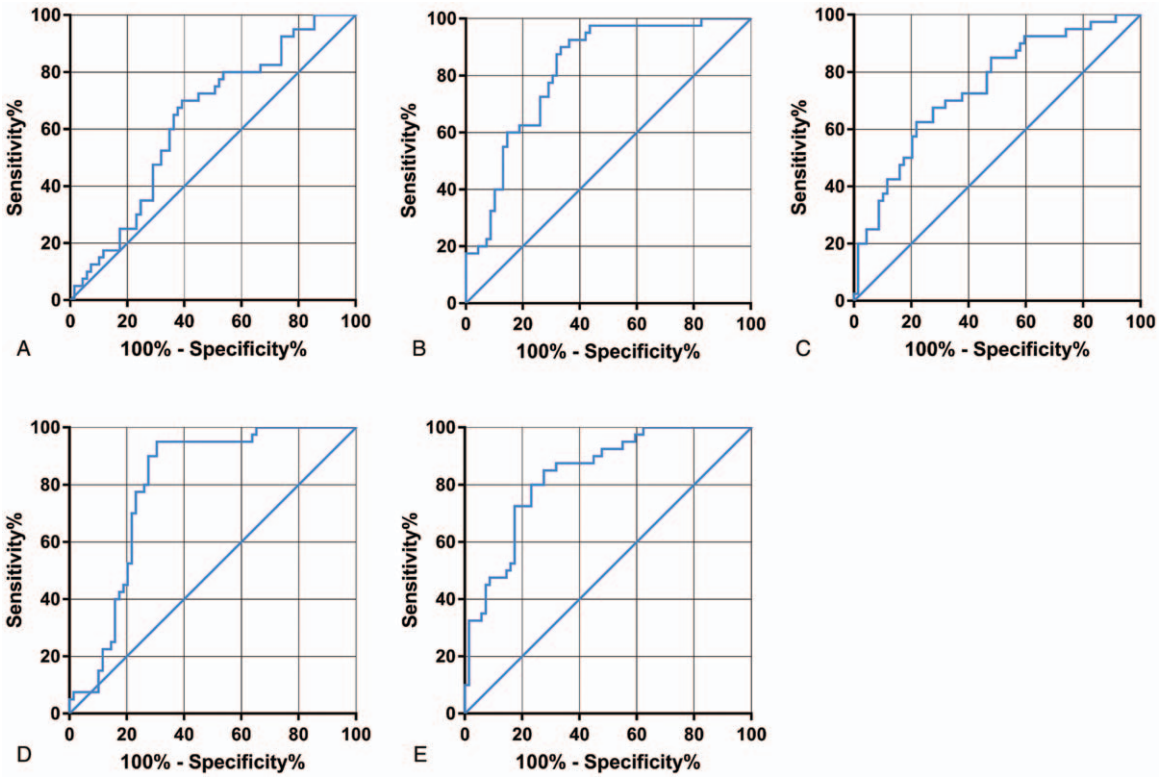

Figure 3. Receiver operating characteristic curve analyses of the diagnostic performance of microRNAs (miRNAs) in identifying patients with severe coronary artery disease. A–E, let-7i-5p, miR-26a-5p, miR-32-3p, miR-3149, and 4-miRNA panel, respectively.

fibrosis by inhibiting unc-51 like autophagy activating kinase 1 expression. Upregulated miR-26a-5p alleviated oxidized LDL-induced apoptosis in aortic endothelial cells treated with kaempferol through inactivation of the toll-like receptor 4/nuclear factor kappa B pathway.[36] Furthermore, miR-26a-5p is downregulated in coronary microembolization, whereas miR-26a-5p overexpression prevented cardiac dysfunction and alleviated myocardial inflammation induced by coronary microembolization.[32] However, miR-26a level is elevated in circulating endothelial cells from patients with atherosclerosis and its overexpression impairs function of endothelial cells.[37,38] AMI patients with stenotic lesions > 70% have higher circulating miR-26a levels, whereas inhibition of miR-26a can protect the mouse heart against ischemic injury.[38] High level of circulating miR-26a-5p is likely to have elevated risk for MI.[20] In the present study, we found that patients with severe CAD had higher plasma miR-26a-5p levels, which might be originated from circulating endothelial cells. These results indicated that miR-26a-5p plays an important role in cardiovascular diseases and may serve as a biomarker in these diseases.

In summary, we identified 4 plasma miRNAs (let-7i-5p, miR-32–3p, miR-3149, and miR-26a-5p) which could serve as novel noninvasive biomarkers for screening patients with severe CAD. The 4-miRNA panel may improve the efficiency of determining the severity of CAD at the time of diagnosis or during follow-up period. Our findings may be beneficial to CAD patients with high risk of major adverse cardiovascular events who have not been considered to receive comprehensive assessment and monitor processes and treatment outcomes of CAD management.

Author contributions

Conceptualization: Xuelin Zhang, Xiaoqiang Li.
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Writing – original draft: Xuelin Zhang, Xiaoqiang Li.
Writing – review & editing: Xiaoqiang Li.

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