Original Paper

Plasma MicroRNA-126-5p is Associated with the Complexity and Severity of Coronary Artery Disease in Patients with Stable Angina Pectoris

Hai-yu Li^a Xin Zhao^b Yu-Zhou Liu^a Zhe Meng^a Dan Wang^a Fan Yang^a
Qiang-wei Shi^a

^aDepartment of Cardiology, The first Affiliated Hospital, Zhengzhou University, Zhengzhou,

^bDepartment of Cardiology, Beijing Anzhen Hospital, Capital Medical University, Beijing, China

Key Words

Atherosclerosis • Biomarkers • Circulating microRNAs • Coronary heart disease

Abstract

Background: Coronary artery disease (CAD) is a major problem worldwide. As an endothelium-enriched microRNA (miRNA), miR-126 has been reported to serve as a potential biomarker of acute myocardial infarction. However, the relationship between miR-126 and the severity of CAD remains unknown. This study was designed to test whether circulating miR-126 levels are associated with the severity of CAD. **Methods:** The present study enrolled 40 patients who had risk factors for CAD without angiographically significant CAD, and 110 patients presenting with stable angina pectoris, who were validated left main coronary artery disease (LMCA) and/or multi-vessel disease by coronary angiography. The expression levels of plasma miR-126-5p from all enrolled subjects were estimated by quantitative real-time polymerase chain reaction (qRT-PCR). Then, the relationships between plasma miR-126-5p levels, number of diseased vessels and the corresponding Synergy between PCI with Taxus and Cardiac surgery (SYNTAX) score were analyzed. **Results:** The expression of circulating miR-126-5p was affected by some CAD risk factors including aging, dyslipidemia and DM. Furthermore, plasma miR-126-5p levels were significantly down-regulated in CAD patients with multi-vessel disease, higher SYNTAX score, rather than isolated LMCA and low SYNTAX score. **Conclusion:** Circulating miR-126-5p has emerged as a potential biomarker for complexity and severity of CAD in patients with stable angina pectoris.

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H.-y. Li, X. Zhao and Y.-Z. Liu contributed equally to this work

Hai-yu Li

Department of Cardiology, The first Affiliated Hospital, Zhengzhou University,
Zhengzhou, He'nan Province, 450000 (China)
Tel. +86-371-67967622, Fax +86-371-67967354, Email lihayu156239@sina.com

Introduction

Coronary artery disease (CAD) represents a major cause of morbidity and mortality worldwide. In recent years, the Synergy between PCI with Taxus and Cardiac surgery (SYNTAX) score was created to predict early and late clinical outcomes, which facilitates to determine the optimal revascularization modality for patients with severe CAD (left main coronary artery disease (LMCA) and/or multi-vessel disease) [1-4]. However, the calculation SYNTAX score is based on angiography results, which is often perceived as difficult and time consuming. Various circulating biomarkers associated with CAD have been identified [5-7], but only few of them provide diagnostic value regarding the severity and complexity of CAD. Therefore, more studies are needed to identify novel biomarkers that assess the severity of CAD.

MicroRNAs (miRNAs) are a class of short (18-22 nt), single-stranded noncoding RNAs that regulate cellular functions through the degradation and translational repression of target mRNAs [8]. miRNAs have been confirmed to be critically involved in various essential biological processes, including proliferation, development, differentiation, and apoptosis [9]. Additionally, circulating miRNAs could be useful as diagnostic or/and prognostic biomarkers for various cardiovascular diseases, such as atrial fibrillation, myocardial infarction (MI), heart failure and cardiac hypertrophy [5, 6, 10-16]. Interestingly, miRNAs have been found in plasma and other body fluids as stable molecules protected from endogenous RNase activity, which suggests that circulating miRNAs might be ideal biomarkers in cardiovascular diseases [17, 18].

Atherosclerosis and aberrant thrombosis are the primary pathological changes involved in CAD [19]. Endothelial dysfunction has been confirmed to be critically involved in the development of atherosclerosis [19]. As an endothelial-enriched miRNA, miR-126 has been reported to play important roles in modulating vascular development and angiogenesis [20]. Additionally, circulating miR-126 in the plasma has been reported to be down-regulated in patients with acute myocardial infarction (AMI) [21]. However, there was still a divergence on the expression of circulating miR-126 in patients with CAD in previous studies, which might be due to the involved patients with different severity and complexity of CAD [22, 23]. Furthermore, the expression level of circulating miR-126-5p in patients with severe CAD remains unknown. So herein, we investigated the expression of miR-126-5p in patients with LMCA and/or multi-vessel disease, and determined the relationship between circulating miR-126-5p levels and the corresponding SYNTAX score of severity and complexity of CAD.

Materials and Methods

Ethics

This study was approved by the Research Ethics Committee of Zhengzhou University. All participants from the First Affiliated Hospital of Zhengzhou University were enrolled between June 2013 and May 2015. All patients involved in this study received oral and written information regarding the objectives of the study and provided written informed consent.

Population study

In total, 110 patients who were scheduled for coronary angiography due to stable angina pectoris (SAP), and subsequently who were diagnosed LMCA or/and multi-vessel disease were enrolled in our study. Another 40 patients matched for sex, age, smoking status, hypertension, and diabetes mellitus (DM) without angiographically significant CAD served as controls. Coronary angiograms were obtained at the catheter-laboratory in the Department of Cardiology at the First Affiliated Hospital of Zhengzhou University. The complexity and severity of the CAD was scored when the following criteria were met: stenosis of $\geq 50\%$ in isolated LMCA or/and multi-epicardial vessels (vessel diameter > 1.5 mm). Qualitative Comparative Analysis software was used (QCA, CAAS, Siemens) to measure the percentage of stenosis and the dimension of the vessel. Additionally, a detailed review of each patient's characteristics and medical history was collected to

gather data regarding medications at time of admission and risk factors for CAD, including age, male gender, smoking status, hypertension, DM and dyslipidemia. To minimize the potential influence of other diseases, the general exclusion criteria included the following: a known history of other cardiovascular diseases (e.g., atrial fibrillation, congestive heart failure, unstable angina pectoris (UAP) and MI), a history of either percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG), severe hepatic or renal dysfunction, recent infection or active chronic inflammatory disease during the last 6 weeks.

Plasma collection and storage

In the present study, peripheral venous blood samples for all participants involved were collected in EDTA-coated tubes when the patients were admitted to the cardiovascular medicine department. All blood samples were then treated with the following protocol: centrifugation at $1500 \times g$ for 10 min at 4°C to remove large cells and debris. The supernatant was then isolated and centrifuged at $14,000 \times g$ for 15 min at 4°C to obtain platelet-poor plasma. All plasma samples were stored in aliquots at -80°C until RNA extraction.

MicroRNA analysis

Total RNA was extracted from plasma using the TRIzol LS Reagent (Invitrogen, US) in accordance with the manufacturer's instructions. After purification from plasma (500 μL), total RNA was eluted into 10 μL of RNase-free water. Subsequently, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the Bulge-Loop™ miRNA qRT-PCR Detection Kit (Ribobio Co., Guangzhou, China) and TransStart™ Green qPCR SuperMix (Ribobio Co., Guangzhou, China) with *Caenorhabditis elegans* miR-39 (cel-miR-39) used as the normalization control. Then, qRT-PCR was performed with the ABI 7500 Fast Real-Time PCR System according to the manufacturer's protocol. The reactions were incubated at 95°C for 30 s, followed by 40 cycles of 95°C for 30 s, 60°C for 20 s, and 70°C for 1 s. Finally, the cycle threshold (Ct) values were normalized to cel-miR-39 using the formula $2^{-(\text{Ct}[\text{miRNA}] - \text{Ct}[\text{cel-miRNA-39}])}$, and the $2^{-\Delta\Delta\text{Ct}}$ method was used to calculate the relative expression level of miR-126-5p in all samples.

SYNTAX score calculation

The SYNTAX scoring system was applied to calculate the severity and complexity of the CAD using the online SYNTAX calculator version 2.11 (www.syntaxscore.com). The SYNTAX score was determined by two experienced interventional cardiologists who were blinded to the patient's clinical and laboratory data. Based on previous reports, all enrolled patients were divided into tertiles according to the corresponding SYNTAX score of CAD as follows: low, 0-22; intermediate, 23-32; high, >33.

Statistical analyses

All data are presented as the means $\pm$ standard deviations (SD) or standard errors (SEM). Continuous variables were compared using either the Mann-Whitney U test or the Kruskal-Wallis test, whereas the one-way ANOVA and Tukey's test were used to compare more than two groups. For categorical variables, either the Chi-Square test or Fisher's test was used appropriately. Univariate linear regression analysis were used to investigate the correlations between the plasma levels of miR-126-5p and CAD traditional risk factors. Additionally, univariate and multivariable linear regression analysis were performed to determine the correlation between plasma miR-126-5p and severity of CAD. For all statistical analyses, SPSS 13.0 was used, and a two-tailed $p < 0.05$ was considered to be significant.

Results

Participant characteristics

The basic characteristics of the patients and control subjects are shown in (Table 1). There were no significant differences among CAD patients with different SYNTAX score and the control group for any of the considered variables (i.e., age, male gender, BMI, smoking status, history of DM, total cholesterol, total triglycerides, LDL cholesterol, HDL cholesterol, heart rate, systolic blood pressure, diastolic blood pressure, creatinine and high sensitivity C-reactive protein). However, the prevalence of dyslipidemia history, SYNTAX score and

Table 1. Baseline characteristics of patients with low, medium and high Syntax score and controls. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, total triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; DM, diabetes mellitus; Hs-CRP, high-sensitivity C-reactive protein; ACEI/ARB angiotensin converting enzyme inhibitor/angiotensin receptor blocker. Data are shown as the mean $\pm$ SD; * $p < 0.05$ and ** $p < 0.01$ for the CAD groups vs. control group

Variables	Controls		CAD		P value
			Syntax Scores		
Participates number	(n = 40)	≤22 (n = 40)	>22 and ≤32 (n = 40)	>32 (n = 30)	
Age (years)	64.0 ± 10.4	66.5 ± 11.7	67.4 ± 9.7	68.9 ± 11.3	0.34
Male Gender, n (%)	28 (70%)	26 (65%)	24 (60%)	17 (57%)	0.66
BMI, kg/m²	23.9 ± 3.5	24.9 ± 2.7	24.4 ± 3.0	25.2 ± 3.2	0.31
Smoker, n (%)	10 (25%)	12 (30%)	12 (30%)	13 (43%)	0.42
Heart rate	68.4 ± 7.8	67.5 ± 9.5	70.4 ± 8.5	72.4 ± 11.8	0.14
Hypertension history, n (%)	12 (30%)	16 (40%)	15 (38%)	14 (47%)	0.55
SBP (mmHg)	128.5 ± 11.5	132.4 ± 13.6	131.2 ± 12.9	129.7 ± 13.4	0.56
DBP (mmHg)	78.2 ± 8.7	80.5 ± 9.4	79.8 ± 10.5	82.0 ± 9.8	0.42
DM, n (%)	5 (13%)	9 (23%)	11(28%)	10 (33%)	0.19
Dyslipidemia history, n (%)	14 (47%)	22 (55%)	26 (65%)	20 (67%)	<0.01
TC (mmol/L)	4.2 ± 0.2	4.4 ± 0.5	4.3 ± 0.6	4.5 ± 0.4	0.21
TG (mmol/L)	1.7 ± 0.9	1.9 ± 0.3	1.9 ± 0.2	2.0 ± 0.2	0.09
HDL-C (mmol/L)	1.0 ± 0.04	1.0 ± 0.04	1.0 ± 0.03	0.98 ± 0.03	0.06
LDL-C (mmol/L)	2.7 ± 0.8	2.6 ± 0.7	2.6 ± 0.4	2.5 ± 0.6	0.65
Creatinine (mg/dl)	68.4 ± 12.3	72.4 ± 16.5	70.7 ± 14.8	75.5 ± 15.6	0.25
Hs-CRP (mg/L)	0.9 ± 1.5	1.5 ± 1.9	1.6 ± 2.1	1.8 ± 3.5	0.36
Syntax score	/	12.4 ± 3.1	26.0 ± 2.6	35.2 ± 2.2	<0.01
Medications on admission					
Aspirin, No. (%)	8 (20%)	30 (75%)	32 (80%)	24 (80%)	<0.01
Clopidogrel, No. (%)	/	7 (18%)	5 (13%)	5 (17%)	<0.01
Beta-blockers, No. (%)	6 (15%)	17 (43%)	22 (55%)	16 (53%)	<0.01
ACEI/ARB, No. (%)	7 (18%)	20 (50%)	25 (63%)	18 (60%)	<0.01
Statins, No. (%)	12 (30%)	21 (53%)	26 (65%)	20 (67%)	<0.01

medication on admission (including aspirin, clopidogrel, beta-blockers, ACEI/ARB and statins) were significantly different between these groups.

Association of plasma miR-126-5p levels and traditional risk factors of atherosclerosis

It is well established that many traditional risk factors (e.g., gender, age, smoking, DM, BMI or hypertension) play important roles in the development of atherosclerosis. To further investigate whether these traditional risk factors impact the expression of circulating miR-126-5p in patients with severe CAD, we performed a univariate linear regression analysis.

The results showed that some risk factors including aging, DM and hyperlipidemia were negatively associated with the down-regulation of circulating miR-126-5p ($p = 0.043$, 0.008

Table 2. Univariate linear regression analysis between traditional cardiovascular diseases and the plasma levels of miRNA-126-5p. BMI, body mass index; DM, diabetes mellitus; Data are shown as the mean $\pm$ SEM; * $p < 0.05$ and ** $p < 0.01$

Variables	Estimate	SEM	P value
Age	-0.004	0.002	0.043*
Male gender	0.087	0.078	0.267
BMI	0.004	0.007	0.552
DM	-0.127	0.072	0.008**
Hypertension	-0.036	0.039	0.351
Dyslipidemia	-0.196	0.076	0.011*
Smoker	-0.006	0.047	0.891

Fig. 1. Association between plasma miRNA-126-5p level and the diseased vessel in severe CAD patients presenting with stable angina pectoris and control subjects. The expression level of plasma miR-126 in control groups and patients with isolated LMCA; LMCA plus single-, double- or triple-vessel disease and triple-vessel disease alone. CAD, coronary artery disease; LMCA, left main coronary artery; CTRL, control group. The data are shown as the mean $\pm$ SD; ** p <0.01. * p <0.05 and ** p <0.01 for the CAD groups vs. the control group.

Fig. 2. Association between plasma miRNA-126-5p level and the SYNTAX score in SAP patients with coronary artery disease. SYNTAX score, Synergy between PCI with Taxus and Cardiac surgery score; L-SYNTAX, low SYNTAX score group; M-SYNTAX, intermediate SYNTAX score group; H-SYNTAX, high SYNTAX score group. The data are shown as the mean $\pm$ SD; * p <0.05 and ** p <0.01 for the CAD groups vs. the control group.

Table 3. Association of miRNA-126-5p with the severity and complexity of coronary artery disease. SYNTAX score, Synergy between PCI with Taxus and Cardiac surgery score. Data are shown as the mean $\pm$ SEM; ** p <0.01

	Univariate			Adjusted for traditional risk factors		
	Estimate	SEM	P value	Estimate	SEM	P value
SYNTAX score	-0.011	0.002	<0.001**	-0.011	0.002	<0.001**

and 0.011, respectively) (Table 2). However, male gender, BMI, hypertension and smoking were not significantly associated with the down-regulation of plasma miR-126-5p levels (p =0.267, 0.552, 0.351 and 0.891, respectively) (Table 2).

Down-regulation of plasma miR-126-5p levels is associated with the severity of CAD

Based on the diseased vessel according to their coronary angiography results, the 110 CAD patients were categorized as follows: 26 patients with an isolated LMCA (24%), 22 patients with LMCA plus single-vessel disease (20%), 20 patients with LMCA plus double-vessel disease (18%), 15 patients with LMCA plus triple-vessel disease (14%) and 27 patients with three-vessel disease (25%). The expression level of miR-126-5p in patients with multi-vessel disease was lower than the control group (Fig. 1). However, the expression of circulating miR-126-5p was not dramatically down-regulated in patients with an isolated LMCA compared with the control subjects.

Association of plasma miR-126-5p levels with the SYNTAX score

To further investigate the association between the expression of plasma miR-126-5p and the severity and complexity of CAD, we determined the association of circulating miR-126-5p with the corresponding SYNTAX score of the diseased vessels. Interestingly, there was no significant difference in the expression of circulating miR-126-5p between CAD patients with low SYNTAX score and control subjects. However, compared to controls, the expression of miR-126-5p was significantly lower in CAD patients with intermediate or high SYNTAX score (Fig. 2). Moreover, the results both of the univariate and multivariate linear regression

analyses after adjustment for traditional coronary risk factors showed that a higher SYNTAX score was significantly associated with lower miR-126-5p levels (estimate, -0.011; standard error, 0.002; $p < 0.001$ and estimate, -0.011; standard error, 0.002; $p < 0.001$, respectively) (Table 3). Meanwhile, plasma miR-126-5p levels were significantly negatively correlated with the SYNTAX score ($r = 0.511$, $p = 0.001$), suggesting that the reduced expression of plasma miR-126-5p is significantly associated with more severe and complex CAD (Fig. 3).

Fig. 3. The correlation between plasma miRNA-126 and the SYNTAX score. SYNTAX score, Synergy between PCI with Taxus and Cardiac surgery score.

Discussion

In the present study, we found that plasma miR-126-5p was significantly down-regulated in patients with severe CAD. Furthermore, some traditional cardiovascular disease risk factors, such as aging, DM and dyslipidemia should be responsible for the down-regulation of plasma miR-126-5p in CAD patients. Compared with control subjects, the expression of plasma miR-126-5p was significantly lower in CAD patients with either intermediate or high SYNTAX score, instead of low SYNTAX score. Additionally, plasma miR-126-5p was significant negative correlation with the SYNTAX score of diseased vessel. According to the above findings, our present study indicated that circulating miR-126-5p might be as a novel predictive biomarker for the severity and complexity of coronary artery lesions in patients with SAP.

Atherosclerosis is currently defined as a chronic inflammatory disease of the vascular wall, which constitutes basic pathology changes of cardiovascular and cerebrovascular diseases, such as MI, ischemic stroke, peripheral arterial disease and carotid plaques [19]. Some substantial changes contribute to the pathological changes of atherosclerosis, including dysfunction of vascular wall cells [i.e., endothelial cells (ECs) and vascular smooth muscle cells (VSMCs)] and the recruitment of leukocytes [24]. According to previous studies, dysfunctional ECs play a pivotal role in atherosclerosis by impairing endothelium-dependent vasodilatation, increasing vascular permeability, up-regulating the expression of chemokines/adhesion molecules and decreasing endothelial regeneration [24]. So, some biomarkers which impact on endothelial function might reflect the condition of atherosclerosis.

In recent years, emerging evidence has indicated that miRNAs play important roles in regulating endothelial function and atherosclerosis [25]. As one of the most abundant miRNAs in ECs, miR-126-5p plays a crucial anti-atherogenic role by regulating the function of ECs and enhancing endothelial repair [26]. miR-126-5p deficiency led to leaky vessels and hemorrhage due to the loss of vascular integrity during embryonic development, which is partly mediated by direct targeting of negative regulators of vascular endothelial growth factor (VEGF) signaling, such as sprout-related proteins and phosphoinositide-3 kinase regulatory subunit 2 [27]. In addition, Harris et al. [28] reported that miR-126-5p negatively regulates leukocyte adherence to ECs by targeting endothelial vascular cell adhesion molecule-1 (VCAM-1) in ECs. A study by Van Solingen et al. showed that transferring EC-derived apoptotic micro-vesicles containing miR-126-5p into atherosclerotic lesions significantly ameliorates the progression of atherosclerosis through the recruitment of Sca-1-positive EC progenitor cells [29]. Meanwhile, miR-126-5p also could promote endothelial proliferation and limit atherosclerosis by suppressing Notch1 inhibitor delta-like 1 homolog (Dlk1) [30].

It has been established that miR-126-5p is significantly down-regulated in human atherosclerotic lesions [30]. Meanwhile, circulating miR-126-5p also is dramatically down-regulated in patients with acute coronary syndrome (ACS), including AMI and UAP [21, 31]. In a recent study, Fichtlscherer et al. reported that plasma miR-126 was down-regulation in patients with CAD [22]. However, Sun et al. found that plasma was not down-regulation in patients with CAD [23]. We assumed that the different plaque burden of diseased vessel in the enrolled participants might be responsible for the discrepancy findings among these studies. Thus, for the first time, we investigated the predictive value of plasma circulating miR-126-5p for the severity and complexity of CAD. Our data indicated that not only miR-126-5p is critically involved in the regulation of EC function and plaque formation, but circulating miR-126-5p levels can predict the severity of atherosclerosis lesions in CAD patients presenting with SAP.

Our data demonstrated that the expression of circulating miR-126-5p could also be influenced by some traditional cardiovascular risk factors, including aging, DM and hyperlipidemia, which is in accordance with previous studies [23, 32-34]. It is worth noting that although these risk factors have been reported to be critically involved in the formation of atherosclerosis, circulating miR-126-5p is a potential independent predictive factor for the severity of cardiovascular artery disease.

In recent years, the SYNTAX score was created to evaluate the severity and complexity of CAD, which facilitates the identification of the optimal strategy for patients with severe CAD [1, 3]. Currently, the optimal strategy for patients with LMCA and/or multiple vessel disease is mainly based on angiography results, which has many clinical advantages [35]. However, one major limitation is that the calculation of the SYNTAX score is often perceived as difficult and time consuming. We are committed to identifying biomarkers that can accurately reflect the severity and complexity of coronary vessel lesion in patients with CAD. An increasing number of biomarkers (e.g., hemoglobin A1, decoy receptor 3 and serum endocan) may serve as independent predictors for CAD and its severity [36-38]. Regarding miRNAs, a recent study by Gao et al. [39] reported that circulating miR-145, as a VSMC-enriched miRNA that plays an important role in atherosclerosis, was negatively associated with the SYNTAX score and the severity of coronary artery lesion in CAD patients. Our present study indicates that miR-126-5p could serve as another potential miRNA biomarker for the severity and complexity of CAD, which might facilitate the identification of optimal treatment strategies for patients with highly complex coronary artery disease. However, we noticed that the expression of miR-126-5p was neither significantly down-regulated in SAP patients with isolated LMCA nor in patients with low SYNTAX score compared to the controls, which might be due to that the down-regulation of circulating miR-126-5p must reach a threshold plaque burden of diseased vessels.

Interestingly, a recent study reported that up-regulation of miR-126 improved cardiac vascular density and function of monocrotaline-induced pulmonary arterial hypertension (PAH) rats [40]. However, in future, more studies should be used to investigate whether up-regulation of circulating miR-126 (*in vivo*) improve endothelial function, stabilize vulnerable plaque, which might provide a potential therapeutic for the severity and complexity of CAD.

Limitations

There may be several limitations in the present study. First, as a single center study with a small sample size, the predictive value should be interpreted with caution. Larger clinical studies are definitely required to determine the clinical diagnosis value of miR-126-5p on the severity and complexity of CAD. Second, more studies should be performed to investigate the underlining mechanisms of the association between down-regulated circulating miR-126-5p and severity of CAD. Third, our present data focus on the predictive value of circulating miR-126-5p for the severity of CAD in patients with SAP. However, more studies are necessary to investigate the divergence expression of miR-126-5p in patients with AMI/UAP and the

severity of CAD in patients with SAP. Finally, there is no doubt that the cardiac/skeletal-driven miRNAs including miR-208, miR-499, miR-1 and miR-133 are critically involved in the CAD, and which have been reported as potential biomarkers for AMI. However, the main pathological changes are endothelial dysfunction and atherosclerotic plaque rather than myocardial necrosis and cell apoptosis in CAD patients presenting with SAP. So, herein, our present study focus on the endothelial-enriched miRNA rather than the widely reported cardiac-specific miRNAs (eg. miR-208, -499, -1 and -133), which clinical value should be investigated by more studies.

Conclusion

Overall, our present study provides the first evidence that plasma miR-126-5p levels are negatively associated with SAP patients who have severe and complex coronary artery disease. This knowledge may ultimately facilitate the selection of an optimal treatment strategy for the patients.

Disclosure Statement

The authors have no disclosures to report.

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