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Original Paper

The Association of Circulating MiR-29b and Interleukin-6 with Subclinical Atherosclerosis

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Key Words
Subclinical atherosclerosis • MicroRNA • MiR-29b • Interleukin-6

Abstract
Background/Aims: Although it is widely acknowledged that atherosclerosis is mainly a chronic inflammatory process, in which both miR-29b and interleukin-6 (IL-6) play multifaceted roles, the association between miR-29b and IL-6 remains unknown. The aim of the present study was to explore the relationship between miR-29b and IL-6 and to test whether circulating levels of miR-29b and IL-6 could predict atherosclerosis. ***Methods:*** A total of 170 participants were divided into two groups according to carotid intima-media thickness (CIMT): study group (CIMT ≥ 0.9mm) and control group (CIMT < 0.9mm). Levels of circulating miR-29b and IL-6 were measured by quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The association of miR-29b and IL-6 levels with CIMT was assessed using Spearman correlation analysis and multiple linear regression analysis. ***Results:*** The study group showed higher miR-29b levels (31.61 ± 3.05 *vs.* 27.91 ± 1.71 Ct, $p < 0.001$) and IL-6 levels (3.40 ± 0.67 *vs.* 2.99 ± 0.37 pg/ml, $p < 0.001$), compared with the control group. CIMT was positively correlated with miR-29b (r = 0.587, $p < 0.001$) and IL-6 (r = 0.410, $p < 0.001$), and miR-29b levels were also correlated with IL-6 (r = 0.242, $p = 0.001$). Multiple linear regression analysis also showed that CIMT was positively correlated with miR-29b and IL-6. After adjustment for age, body mass index, systolic blood pressure, total cholesterol and C-reactive protein, CIMT was still closely correlated with miR-29b and IL-6. The combination of miR-29b and IL-6 (AUC = 0.901, $p < 0.001$) offered a better predictive index for atherosclerosis than either miR-29b (AUC = 0.867, $p < 0.001$) or IL-6 (AUC = 0.747, $p < 0.001$) alone. ***Conclusion:*** Circulating levels of miR-29b and IL-6 may be independently correlated with subclinical atherosclerosis, and may serve as novel biomarkers for the identification of atherosclerosis.

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Introduction

Atherosclerosis is the leading cause of cardiovascular disease (CVD), which is a major global public health problem in modern society [1]. Atherosclerosis is a condition in which an accumulation of cells, cholesterol and extracellular matrix causes thickening and hardening of the arterial wall [2]. Measurement of carotid intima-media thickness (CIMT) is routinely used to assess subclinical or clinical atherosclerosis and current evidence suggests that CIMT may indicate subclinical vascular disease that is amenable to preventative measures [3]. CIMT has been shown to predict cardiovascular risk in many large studies and is recommended for cardiovascular risk assessment in asymptomatic adults at intermediate risk of CVD [4]. It is now generally believed that atherosclerosis is mainly a chronic inflammatory process and increasing numbers of inflammatory mediators have been shown to play vital roles in the development of atherosclerosis [2].

Circulating microRNAs (miRNAs, miRs) are a type of non-coding RNA, about 21–25 nucleotides in length, which play vital roles in the modulation of gene expression at the post-transcriptional level [5]. It has been demonstrated that miRNAs contribute to a variety of biological processes, including angiogenesis or vascular remodeling, inflammation and oxidative stress [6]. miR-29b is a key circulating cytokine, which plays a multifaceted and vital role in atherosclerosis [7]. IL-6 is another cytokine that has previously been shown to have pro-inflammatory effects and to play an important role in propagating the downstream inflammatory response that underlies atherosclerosis [8].

There are, however, limited date on the relationship of IL-6 and miR-29b with subclinical atherosclerosis and there are also no data on the levels of these biomarkers that would indicate subclinical atherosclerosis. In the present study, we sought to explore whether circulating levels of miR-29b and IL-6 might represent potential non-invasive biomarkers of subclinical atherosclerosis.

Materials and Methods

Between May 2015 and September 2016, all the participants were enrolled consecutively in this study from the Guangdong General Hospital, Guangdong, China. A total of 170 participants were enrolled in this study: 85 patients (41 men; mean age, 50.48 ± 5.78 years) with CIMT ≥ 0.9mm as our study group and 85 healthy volunteers (46 men; mean age, 51.22 ± 5.42 years) with CIMT < 0.9 mm as control group. The diagnosis of subclinical atherosclerosis was based on the value of CIMT, which was measured by carotid artery ultrasound. Participants with a history of coronary heart disease, cerebrovascular disease, diabetes mellitus, neck surgery, thyroid disease, heavy smoking or current use of certain medications (lipid-lowering, antiplatelet or antihypertensive drugs) were excluded. Among those exclusion criteria, cerebrovascular diseases were defined as a documented history of ischemic cerebrovascular event with or without sequelae, transient ischemic attack, or amaurosis fugax in the last 6 months. Healthy control subjects were invited from the community or outpatient. All of the participants underwent physical examination, office blood pressure measurement and CIMT measurement before the study. The CIMT of the common carotid artery was measured by ATL HDI 3000 ultrasound system (Advanced Technology Laboratories, Bothell, WA) equipped with a 5-MHz linear array transducer as previously described [9]. A value < 0.9 mm was defined as normal CIMT and a value ≥ 0.9 mm was defined as increased CIMT. Subclinical atherosclerosis was defined as CIMT ≥ 0.9 mm [10]. The study was approved by the Medical Ethics Committee of Guangdong General Hospital and written informed consent was obtained from each patient prior to participation. The study was conducted in accordance with the Declaration of Helsinki.

Sample Collection

Samples were collected in the morning, prior to any treatment, from patients and healthy volunteers, following an overnight fast. Plasma was extracted by centrifuging blood samples at 3000 rpm for 10 min at room temperature. Plasma samples were collected and divided into two aliquots, which were frozen separately at −80°C before being used for analysis. Fasting blood glucose, serum lipid profiles, routine laboratory tests and renal function were measured using routine methods.

Detection of miR-29b and IL-6

Total RNA, containing small RNAs, was extracted from plasma using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using a mirVana miR Isolation Kit (Ambion, Austin, TX, USA), according to the manufacturer's protocol [11]. Plasma miRs were evaluated using the S-Poly (T) RT-qPCR method, as previously described, with miR-54 as the control [12]. The comparative cycle threshold (Ct) (ΔCt) was used to calculate the relative level of miRs. Mean Ct values and deviations between duplicates were calculated for all samples. ΔCt = Ct (miR)-Ct (miR-54). Plasma IL-6 levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (QuantiGlo, R&D Systems, Minneapolis, Minnesota), according to the manufacturer's instructions [13].

Statistical analysis

All continuous variables were expressed as mean ± standard deviation. The Mann-Whitney U test or Student *t* test was used to compare continuous variables between patients and controls. Correlations between continuous variables were assessed using the Spearman correlation coefficient. Multiple linear regression analysis was used to assess the relationship between CIMT and miR-29b or IL-6. Adjustments were made for age, body mass index, systolic blood pressure, total cholesterol and C-reactive protein (CRP). Statistical significance was defined as two-sided $p < 0.05$. The predictive value of being diagnosed with atherosclerosis was evaluated using the receiver operating characteristic (ROC). Area under the ROC curve (AUC) was used as an accuracy index for evaluating the diagnostic performance of miR-29b and IL-6. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Results

Clinical characteristics, plasma IL-6 concentration and miR-29b levels

Baseline date are shown in Table 1. At baseline, there were no statistically significant differences in age, sex, body mass index, fasting blood-glucose, total cholesterol, high-density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, glomerular filtration rate, diastolic blood pressure or heart rate between the groups. There were, however, statistically significant differences in CRP, systolic blood pressure, IL-6 and miR-29b between the two groups. The study group showed higher miR-29b levels (31.61 ± 3.05 *vs.* 27.91 ± 1.71 Ct, $p < 0.001$) and IL-6 levels (3.40 ± 0.67 *vs.* 2.99 ± 0.37 pg/ml, $p < 0.001$), compared with the control group (Fig. 1A and 1B).

Correlation of miR-29b and IL-6 with CIMT

As shown in Table 2, CIMT was positively correlated with miR-29b (r = 0.587, $p < 0.001$), IL-6 (r = 0.410, $p < 0.001$) and CRP (r = 0.447, $p < 0.001$). miR-29b levels were positively correlated with IL-6 (r = 0.242, $p = 0.001$) and CRP (r = 0.356, $p < 0.001$). IL-6 levels were positively associated with CRP (r = 0.378, $p < 0.001$). Multiple

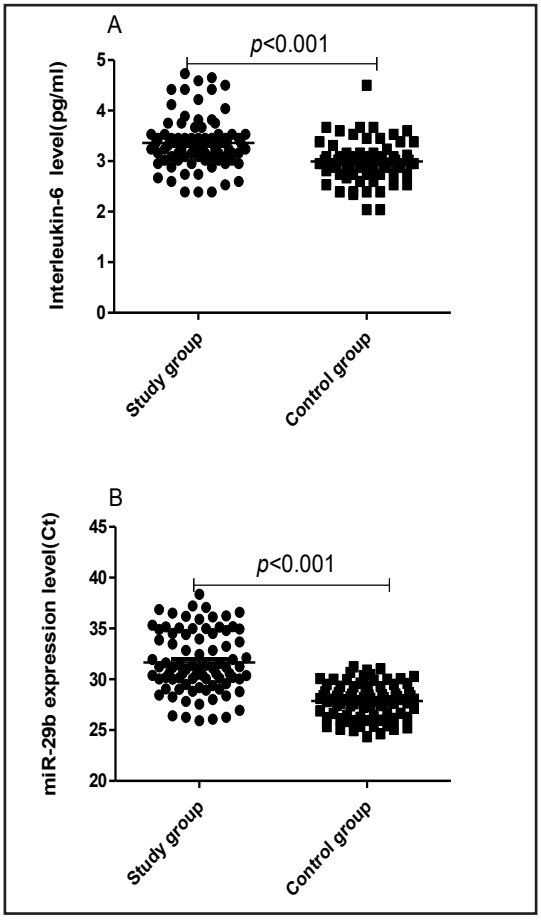


Fig. 1. A. Interleukin-6 levels in study group and control group; B. miR-29b expression levels in study group and control group.

Table 1. Demographic, clinic and laboratory characteristics between groups. HDL-C, high-density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; GFR, glomerular filtration rate; CRP, C-reactive protein; DBP ,diastolic blood pressure; SBP, systolic blood pressure; IL-6,interleukin-6. Data are presented as the mean ± standard deviation

	Study group (n=85)	Control group (n=85)	*p-value*
Males/females	41/44	46/39	0.105
Age (years)	50.48±5.78	51.22±5.42	0.470
Body mass index (kg/m^2)	26.54±3.42	26.85±3.80	0.411
Total cholesterol (mg/dl)	200.40±45.72	192.94±38.00	0.094
HDL-C(mg/dl)	50.46±13.15	49.30±11.25	0.711
LDL-C(mg/dl)	127.16±33.71	123.41±33.52	0.839
Triglyceride (mg/dl)	179.64±55.84	153.86±59.74	0.186
GFR(ml/min/1.73m^2)	111.32±11.65	111.06±13.85	0.278
Fasting blood-glucose(mmol/l)	4.92±0.49	5.01±0.53	0.349
Heart rate(beats/minute)	74.69±10.82	74.89±9.53	0.125
SBP(mm Hg)	141.02±12.05	136.55±15.66	<0.001
DBP(mm Hg)	84.95±8.49	83.42±9.23	0.328
CRP(mg/l)	17.34±5.48	6.83±2.89	<0.001
IL-6(pg/ml)	3.40±0.67	2.99±0.37	<0.001
miR-29b expression level (Ct)	31.61±3.05	27.91±1.71	<0.001

Table 2. Relationship of miR-29b expression level with interleukin-6 and CIMT. CIMT; carotid intima-media thickness; IL-6, interleukin-6; CRP, C-reactive protein. The Spearman correlation coefficient was used for statistical analysis

	miR-29b		IL-6		CIMT	
	r	*p*	r	*p*	r	*p*
miR-29b			0.242	0.001	0.587	<0.001
IL-6	0.242	0.001			0.410	<0.001
CRP	0.356	<0.001	0.378	<0.001	0.447	<0.001

Table 3. Relation of interleukin-6 and miR-29b with subclinical atherosclerosis. IL-6, interleukin-6. Multivariate linear regression analysis was used for statistical analysis. Model1: It was a standard model, Model2: Age, BMI, SBP, TC, and CRP were adjusted

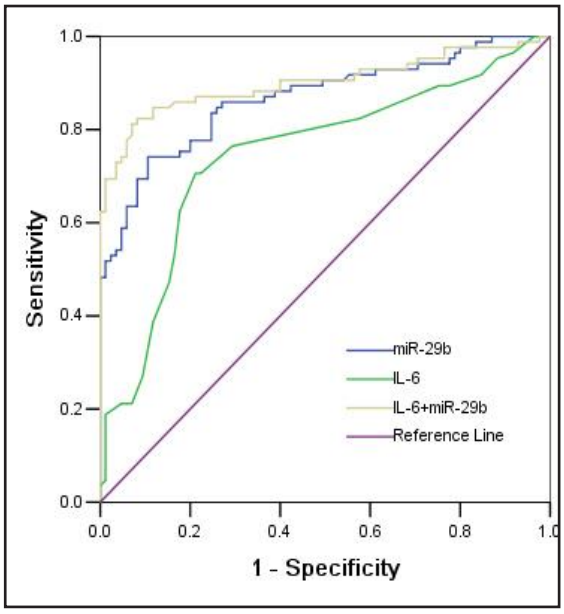
		β	*p*	95%CI
Model1	miR-29b	0.481	<0.001	0.380,0.662
	IL-6	0.232	<0.001	0.154,0.503
Model2	miR-29b	0.467	<0.001	0.372,0.645
	IL-6	0.213	<0.001	0.161,0.407

linear regression also showed that CIMT was positively correlated with miR-29b (β = 0.481, 95% CI 0.380, 0.662; $p < 0.001$) and IL-6 (β = 0.232, 95% CI 0.154, 0.503; $p < 0.001$). After adjustment for age, body mass index, systolic blood pressure, total cholesterol and CRP, CIMT was still closely correlated with miR-29b (β = 0.467, 95% CI 0.372, 0.645; $p < 0.001$) and IL-6 (β = 0.213, 95% CI 0.161, 0.407; $p < 0.001$).

Predictive value of miR-29b and IL-6 for subclinical atherosclerosis

An ROC analysis was performed to determine the predictive values of miR-29b and IL-6 for subclinical atherosclerosis (Table 3). The combination of miR-29b and IL-6 (AUC = 0.901, sensitivity = 95.2%, specificity = 85.1%, $p < 0.001$) was a better predictor for atherosclerosis than either miR-29b (AUC = 0.867, sensitivity = 92.2%, specificity = 81.2%, $p < 0.001$) or IL-6 (AUC = 0.747, sensitivity = 82.3%, specificity = 67.1%, $p < 0.001$) alone (Fig. 2).

Fig. 2. ROC curve for subclinical atherosclerosis.



Discussion

Atherosclerotic diseases are now a major global public health problem and increased CIMT is a hallmark of atherosclerotic diseases [14]. In the present study, subclinical atherosclerosis patients had significantly higher miR-29b, IL-6 and CRP levels than healthy volunteers. CIMT was shown to be positively correlated with miR-29b, IL-6 and CRP; miR-29b levels were positively correlated with IL-6 and CRP; and IL-6 levels were positively correlated with CRP. Importantly, after adjustments for age, body mass index, systolic blood pressure, total cholesterol and CRP, CIMT was still closely correlated with miR-29b and IL-6. An important finding of the study is that either miR-29b or IL-6 would be a good new biomarker for the identification of subclinical atherosclerosis. The most significant and meaningful discovery of this study is that the combined AUC of miR-29b and IL-6 is a better indicator of preclinical atherosclerosis than the AUC of either miR-29b or IL-6 alone.

It is widely acknowledged that atherosclerosis is a complex inflammatory process [2] and, recently, circulating miRNAs have been recognized as novel biomarkers and potential therapeutic targets for CVD, including atherosclerosis [15]. miR-29b, a key circulating cytokine, has been shown to play a multifaceted and vital role in atherosclerosis [7]. We have shown that miR-29b levels are closely correlated with CRP and CIMT. CRP is an important systemic inflammatory marker, which is widely used in the clinic [16] and which has been shown to be predictive for CVD [17]. miR-29b may thus also be a vital inflammatory marker that plays an important role in the development of subclinical or clinical atherosclerosis. miR-29b has also been reported to be associated with endothelial cell dysfunction [18]. Mott et al. [19] reported that miR-29b is an important endogenous regulator, which plays a role in endothelial cell apoptosis by inhibiting the anti-apoptotic gene, myeloid cell leukemia-1. Anke and his team also showed that miR-29b mediates the deposition of collagen and fibrosis via IL-6 and tumor growth factor-β [20]. miR-29b has also been suggested to suppress the proliferation and migration of vascular smooth muscle cells, possibly through inhibition of myeloid cell leukemia-1 and matrix metalloproteinase-2, indicating that miR-29b may serve

as a valuable therapeutic tool to treat CVD, such as atherosclerosis [21].

Atherosclerosis as a common chronic process and many cytokines such as endothelial cells, smooth muscle cells or macrophages, could affect different stages in its progression [2, 22]. IL-6 is an important pro-inflammatory cytokine that may play a leading role in modulating the hepatic synthesis of CRP and fibrinogen [23]. IL-6 is also an upstream regulator that plays a central role in propagating the downstream inflammatory response responsible for atherosclerosis [2]. We found that levels of IL-6 were positively correlated with CRP and CIMT and, after adjustment for age, body mass index, systolic blood pressure, total cholesterol and CRP, CIMT was still closely correlated with IL-6. This indicates that IL-6 is also a risk factor for subclinical or clinical atherosclerosis. Some previous cohort studies showed that patients with high levels of IL-6 had an increased atherosclerotic burden and higher risk of major adverse cardiac events compared with individuals with lower levels of IL-6 [24, 25]. Interestingly, Bacchiega et al. found that the IL-6-blocking agent tocilizumab significantly reduced CRP concentrations, together with parameters of systemic inflammation [26]. IL-6 has many other functions, including activation of macrophages [27, 28] and stimulation of endothelial [29, 30] and vascular smooth muscle cells [31, 32]. Although IL-6 and miR-29b both have a number of clear effects on different stages in the formation of atherosclerosis and are, individually, important markers for CVD, we have shown that that the combination of miR-29b and IL-6 offers a better predictive marker for atherosclerosis than either miR-29b or IL-6 alone. As indicated by our results, circulating levels of miR-29b or IL-6 may serve as underlying and ponderable biomarkers for detecting subclinical or clinical atherosclerosis.

Several limitations should be taken into account when interpreting our results. Firstly, this was a cross-sectional study, which only shows a relationship but does not identify causality, so the results should not be used to draw causal conclusions. Secondly, the number of study subjects was relatively small, and multicenter, prospective studies with larger sample sizes are needed to confirm our results. Thirdly, the underlying mechanisms responsible for the correlation of circulating miR-29b and IL-6 with CIMT were not elucidated. The potential roles of miR-29b and IL-6 in atherosclerosis thus need to be fully elucidated by further basic research.

Conclusion

Our study showed that levels of circulating miR-29b and IL-6 were increased in subjects with subclinical atherosclerosis, compared with healthy individuals. Circulating miR-29b and IL-6 were independently correlated with CIMT. Increased levels of circulating miR-29b and IL-6 may serve as new biomarkers for subclinical or clinical atherosclerosis and may also be useful for clinical monitoring of the early stages in the development of atherosclerosis.

Disclosure Statement

The authors have no competing interests.

Acknowledgements

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