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Circulating miR-214 is associated with the severity of coronary artery disease

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Abstract

Objective   To study whether miR-214 is regulated in coronary artery disease (CAD) patients and whether placental growth factor (PLGF) is a possible target for miR-214 in atherosclerosis. **Methods**   Circulating miR-214 was measured by quantitative PCR using RNA isolated from 40 patients with CAD, including 12 with stable angina pectoris, 16 with unstable angina pectoris and 12 with acute myocardial infarction, and 15 controls without CAD. Plasma level of PLGF was measured by ELISA. **Results**   The miR-214 level was significantly lower in CAD patients compared with that in controls ($P < 0.01$). Compared to controls, patients with unstable angina pectoris (UAP, 38.6 ± 9.1 pg/mL) and acute myocardial infarction (AMI, 46.3 ± 13.4 pg/mL) had significantly higher level of plasma PLGF, but not those with stable angina pectoris (SAP; $P = 0.012$, UAP *vs*. Control; $P = 0.005$, AMI *vs*. Control). In patients with AMI, the plasma level of miR-214 was positively correlated to that of PLGF. **Conclusions**   The results suggest that miR-214 is a beneficial microRNA for CAD patients. Loss of its protection may lead to increased PLGF levels and worsening atherosclerosis. Circulating miR-214 is a promising biomarker for alerting severe CAD.

1   Introduction

Coronary artery disease (CAD) is still a leading cause of death around the world. Recent studies have revealed that microRNA (miRNA) plays an indispensable role in cardiac function through their repression of target mRNA.[1] miRNAs exert their repressive functions by binding to sequences in the 3'-UTRs of target mRNAs that are complementary to nucleotides 2–8 of the miRNA, known as the seed region. However, a variety of cells secrete these small RNAs into extracellular fluids by exosomes or microparticles.[2] The double layered structures make miRNAs remarkably stable and able to survive the degradation of RNases. Furthermore, either placing the unfrozen sample at room temperature for over four hours or repeated freeze-thaw cycles does not substantially change the level of microRNAs.[3] This mechanism provides a new stable source of biomarkers to study clinical diseases and pathological states.

Circulating miR-214 has been shown to be regulated during cardiac hypertrophy and heart failure.[4] Although expression of different circulating miRNAs in CAD patients has been reported,[5] the role of miR-214 in atherosclerosis has not been studied. Here, we explored the level of circulating miR-214 and placental growth factor (PLGF) in CAD patients.

2   Methods

2.1   Study population and samples

The patients were admitted to the Department of Cardiology, Second Military Medical University of Shanghai, China, from November 2011 to November 2012 for coronary angiography (CAG) due to suspected CAD. All of them underwent first-time CAG. The CAD study group consists of 12 patients with stable angina pectoris (SAP), 16 with unstable angina pectoris (UAP) and 12 with acute myocardial infarction (AMI). Diagnosis of SAP and UAP were based on typical chest pain,[6] and angiographically proven CAD, while AMI patients also had increased troponin T. The control group included 10 age-matched people, whose CAG ruled out CAD, and five healthy young volunteers. Exclusion criteria included: a known history of autoimmune disease, acute or chronic infection, severe hepatic or renal dysfunction, and evidence of malignant disease. In patients with AMI, a venous blood sample of 5 mL was

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obtained at the same time of the clinical emergency blood test, while in other patients blood samples were obtained in the next morning after admission.

2.2　The Syntax score and angiographic analysis

Based on the baseline diagnostic angiogram, each coronary lesion producing ≥ 50% diameter stenosis in vessels ≥ 1.5 mm was scored separately. These scores were added together to provide the overall Syntax score, which was calculated using the Syntax score algorithm.[7] The Syntax score of patients was independently assessed by two experienced interventional cardiologists who were blinded to our experiment data. The average value from the two readers was adopted as the final value.

2.3　RNA isolation

Circulating RNAs were isolated from EDTA-plasma from all subjects using a Qiazol-based miRNA isolation protocol. We used a miRNA kit provided by Qiagen (miR-Neasy, Hilden, Germany) which combines phenol/guanidine-based lysis of samples and silica membrane-based purification of total RNA (>18 nucleotides). A total of 200 μL of EDTA-plasma from each subject was used for subsequent studies.

2.4　Detection and quantification of miRNAs by qPCR

Five microlitre out of 50 μL of total RNA, obtained as outlined above, was transcribed reversely using the Taqman microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the instructions of manufacturer. A 2.5 μL of the product was used for detecting

miRNA expression by quantitative PCR using Taqman microRNA Assay kits (ABI). The miR-214 values were normalized to U6 and were expressed as $\Delta CT = CT$ (miR-214)-CT(U6).

2.5　Measurement of PLGF

Plasma level of PLGF was measured by an ELISA kit provided by R&D Systems (Minneapolis USA). Reading of optical density was set at 450 nm and normalized to 570 nm according to the instruction by the manufacturer.

2.6　Statistical analysis

Difference in circulating miR-214 between whole CAD group and control group was examined by Student's *t* test. ANOVA was used to explore any difference in miR-214 among the subgroups of CAD patients. Subsequent differences between groups were analyzed using a doubled-sided Student's *t* test. Experimental data are expressed as mean ± SE of at least three separate experiments. Pearson's correlation was used to explore the relationship between levels of miR-214 and PLGF. The miR-214 expression level was also assessed for correlation with patients' maximum coronary stenosis, number of diseased vessels, ejection fraction and cholesterol level using Spearman test. A value of $P < 0.05$ was considered statistically significant. Statistical analysis was done using SPSS 18.0.

3　Results

3.1　Patient characteristics

Patient characteristics are shown in Table 1. The median

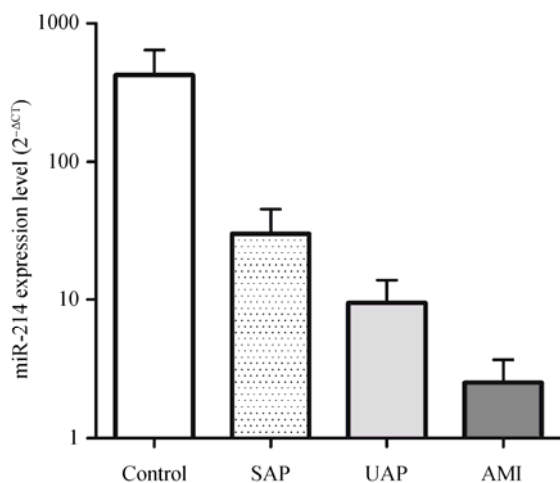**Table 1.　Patients characteristics.**

Characteristic	All	Control	CAD
Total number	55	15	40
Male (%)	36 (65.5)	6 (40)	30 (75)
Age (SE)	63.4 (14.1)	54.3 (17.5)	66.8 (11.1)
Diabetes mellitus (%)	7 (12.7)	2 (13.3)	5 (12.5)
Hypertension (%)	31 (56.4)	4 (26.7)	27 (67.5)
Current smoker (%)	28 (50.9)	4 (26.7)	24 (60)
Total cholesterol (mmol/L)	3.89 (0.91)	3.86 (0.66)	3.90 (0.99)
LDL-C (mmol/L)	2.28 (0.68)	2.05 (0.39)	2.37 (0.74)
HDL-C (mmol/L)	1.05 (0.28)	1.07 (0.28)	1.04 (0.29)
Triglyceride (mmol/L)	1.64 (0.73)	1.94 (0.67)	1.52 (0.73)
Glucose (mmol/L)	6.07 (2.14)	5.53 (1.15)	6.28 (2.39)
Ejection fraction (%)	73.7 (8.3)	77.1 (7.5)	72.5 (8.4)
Syntax score			
Low (0-22)	51 (92.7)	15 (100)	36 (90)
Intermediate (23-32)	2 (3.6)	0 (0)	2 (5)
High (33 or more)	2 (3.6)	0 (0)	2 (5)

CAD: coronary artery disease patients; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol.

age was 63 years old; 36 (65.5%) were male, 7 (12.7%) had diabetes mellitus and 31 (56.4%) had hypertension. The CAD patients with low (0–22), intermediate (23–32), and high (33 and over) Syntax score were 51 (92.7%), 2 (3.6%), and 2 (3.6%), respectively.

3.2 Plasma miR-214 levels in CAD patients versus control group
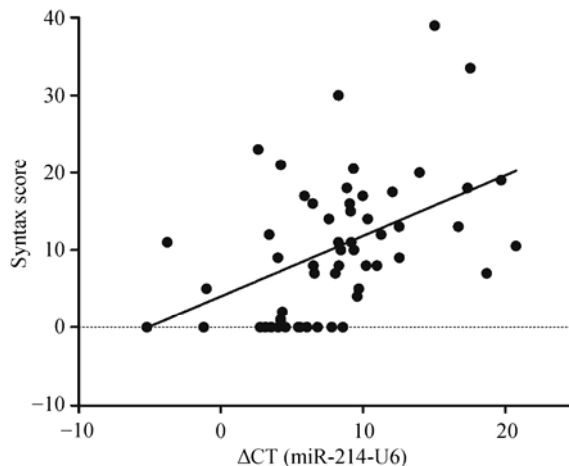
As shown in Figure 1, all the CAD subgroups had significantly decreased expression of miR-214 when compared wth the control group. Within the CAD subgroups, patients with AMI appeared to have the lowest level of circulation miR-214, although there were no statistical differences among the three subgroups. In addition, the syntax score was correlated with plasma miR-214 level (Spearman's rank correlation coefficient; $r = -0.468$, $P < 0.01$) (Figure 2).

Figure 2. Correlation between the Syntax score and plasma level of miR-214. ($n = 55$, Spearman's $r = 0.468$, $P < 0.01$).
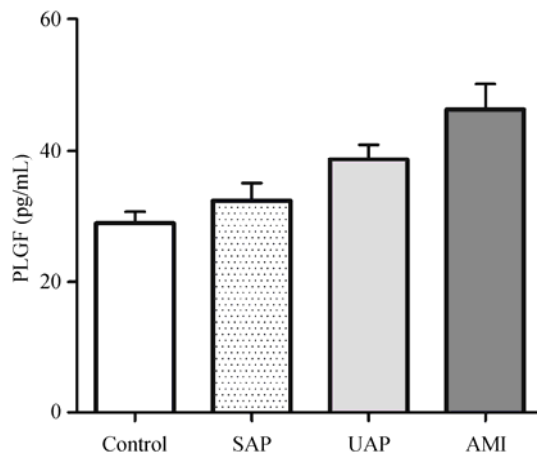
Figure 1. Circulating miR-214 levels in controls and CAD sub-groups. miR-214 was downregulated in CAD patients (all $P <$ 0.05 as compared to controls; $P > 0.05$ when compared among SAP, UAP and AMI sub-groups). AMI: acute myocardial infarction; CAD: coronary artery disease patients; SAP: stable angina pectoris; UAP: unstable angina pectoris.

Figure 3. Plasma levels of PLGF in CAD patients and controls. Compared to controls, patients with UAP and AMI had significantly higher level of plasma PLGF. ($P = 0.86$, SAP *vs*. Control; P = 0.012, UAP *vs*. Control; $P = 0.005$, AMI *vs*. Control). AMI: acute myocardial infarction; CAD: coronary artery disease patients; PLGF: placental growth factor; SAP: stable angina pectoris; UAP: unstable angina pectoris.

3.3 Plasma PLGF levels in CAD patients versus control group

The mean level of plasma PLGF was 32.3 ± 9.3 pg/mL in patients with SAP, which was not significantly different from the control group (28.9 ± 6.6 pg/mL, $P = 0.86$); However, compared with controls, patients with UAP (38.6 ± 9.1 pg/mL) and AMI (46.3 ± 13.4 pg/mL) had a significantly higher level of plasma PLGF ($P = 0.012$, UAP *vs*. Control; $P = 0.005$, AMI *vs*. Control). These results were depicted in Figure 3.

3.4 Correlation of plasma miR-214 and PLGF levels in CAD patients and controls

In patients with AMI, the miR-214 level was correlated to the PLGF expression (Figure 4), but not in other individual group.

4 Discussion

This study demonstrates that circulating miR-214 can be detected in the blood and is down-regulated in patients with
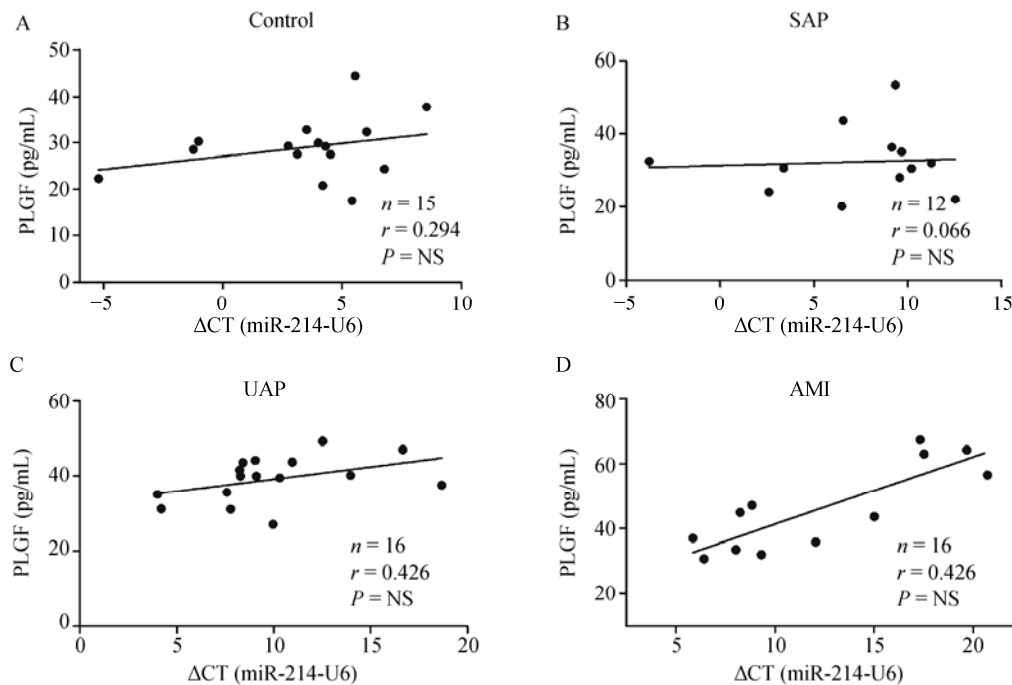
Figure 4.   Correlation between PLGF and miR-214 in CAD patients. (Pearson *r* = 0.669, *P* < 0.01). AMI: acute myocardial infarction; CAD: coronary artery disease patients; NS: no significant; PLGF: placental growth factor; SAP: stable angina pectoris; UAP: unstable angina pectoris.

CAD. Furthermore, miR-214 tends to decrease to a lower level when patients suffered from a more severe form of CAD. However, the reduced expression level is only associated with the maximum stenosis of the coronary artery, but not the total number of diseased vessels. Moreover, the miR-214 level was not correlated to ejection fraction or blood cholesterol level. Taken together, these results indicate that miR-214 might be a promising biomarker as an alert of vulnerable plaque, rather than impaired cardiac function, or metabolic state.

miR-214 has been shown to be closely related to eNOS and, hence, angiogenesis.[8] And intra-plaque angiogenesis is proposed as a mechanism of atherosclerosis progression. Due to this relationship, PLGF, which promotes angiogenesis and has been reported to be associated with the symptoms[9] and prognosis[10] of atherosclerosis, was measured to examine any correlation with miR-214. The result in AMI patients partially confirmed this hypothesis, but it only means miR-214 and PLGF were inversely regulated at the same time during myocardial infarction. The substantial regulatory role of miR-214 and PLGF expression requires further investigation and it remains to be defined in which cells or tissue they are possibly influenced.

In addition, the reduced level of circulating miR-214 was thought provoking because endothelial activation, plaque rupture, or myocardial death were always expected in patients with AMI, thus, the level of circulating miRNAs would be elevated. In mice, miR-214 expression in cardiac myocyte was up-regulated during ischemic injury, but its change in circulating level was not examined.[11] However, recent studies demonstrate that miRNAs can be delivered by apoptotic bodies to atherosclerotic lesion.[12] In addition, miRNAs can also be transferred between different cells by exosomes[13] or lipoproteins.[14] Therefore, it might be speculated that the reduction of circulating miR-214 may possibly be caused by cellular uptake or accumulation into the atherosclerotic lesions, but this specific cycling circle in CAD patients is completely unclear.

One limitation of the present study is that we cannot provide the molecular insights into the cause of dysregulation. The level of circulating miRNAs may be affected by multiple parameters, such as the change in expression in the tissue, the release of the miRNAs by the cells into the circulation, and the uptake by targeted tissue or cells. A certain miRNA can also be regulated by an overall miRNAs processing since the cellular stress can affect the expression of the enzyme Dicer, which is essential for the biogenesis of mature miRNAs.[15] It is necessary for further studies to explore the mechanism of how CAD and therapy affect tissue versus circulating miR-214 at the same time.

In summary, the present study shows that a new circulating microRNA, miR-214, is down regulated in patients with

CAD and correlated well with the severity of coronary stenosis, which indicates miR-214 as a promising biomarker for CAD. Moreover, the reciprocal regulation of miR-214 and PLGF suggests PLGF might be a repression target of miR-214 during AMI. However, these data surely need to be confirmed in larger clinical populations. And prospective large-scale studies are required to determine the potential use of miR-214 as a biomarker for the development of CAD.

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