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MicroRNA-21 is a unique signature associated with coronary plaque instability in humans by regulating matrix metalloproteinase-9 via reversion-inducing cysteine-rich protein with Kazal motifs

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ABSTRACT

Background: Coronary atherosclerotic unstable plaque is one of the leading causes of cardiovascular death. Macrophage-derived matrix metalloproteinase (MMP) 9 is considered for degrading extracellular matrix and collagen, thereby thinning the fibrous cap in plaques. miR-21 is implicated to play an important role in the progression of atherosclerosis. Nevertheless, miR-21 as the biomarker for coronary atherosclerotic unstable plaque remains unknown. We aimed to investigate the prediction role of miR-21 for unstable plaque by pathway study of miR-21 on MMPs and its inhibitor RECK in macrophages.

Methods: Expression of miR-21 in macrophages and serum miR-21 as well as MMP-9 was measured in patients with coronary non-calcified plaque, calcified plaque and controls. In vitro experiment was done in human macrophages by over-expressing miR-21 or down-regulating RECK. The regulation of RECK and MMP-9 by miR-21 was evaluated by western blotting and siRNA strategy.

Results: Patients with non-calcified coronary artery lesions had significantly higher miR-21 in macrophages and lower miR-21 serum levels compared to the control and calcified plaque patients. At the same time, the serum levels of MMP-9 were significantly elevated in non-calcified patients. Experiments in vitro indicated that over-expressing miR-21 could induce the expression and secretion of pro-MMP-9 and active-MMP-9 in human macrophages via targeting gene RECK, and knocking down RECK expression by specific siRNA can resemble that of miR-21 over-expression.

Conclusions: miR-21 might be a biomarker for plaque instability by suppressing target gene RECK to promote the expression and secretion of MMP-9 in macrophages.

Introduction

Coronary atherosclerotic unstable plaque is one of the leading causes of cardiovascular death globally, but there is still no satisfactory solution in the recognition of high-risk cardiovascular patients in clinical work (Raitoharju et al., 2013). A recent study shows that thickness of the fibrous cap and macrophage infiltration are important discriminators of plaque types (Narula et al., 2013). Matrix metalloproteinase (MMP)—mainly MMP-9—is primarily responsible for degrading extracellular matrix and collagen, thereby thinning the fibrous cap in plaques (Galis et al., 1994). MMP-9 is regulated tightly at two levels: secreted in a zymogenic form (92 kDa) (pro-MMP-9) following proteolytic cleavage yielding the active form (85 kDa) (active-MMP-9) to avoid destructive activity (Nagase, 1997). It has been suggested that unstable (normally non-calcified) plaques undergo thinning of the fibrous cap prior to rupture, possibly as a result of macrophages releasing proteolytic matrix-degrading enzymes which may degrade the fibrous cap (Finn et al., 2010).

MicroRNAs (miRNAs) are endogenous, non-coding and small (18–22 nucleotides) RNA molecules. They regulate gene expression at the posttranscriptional level by imperfect base pairing with the 3′-untranslated region (UTR) of target mRNA, leading to translation repression and/or mRNA degradation (Bartel, 2004). By influencing

Abbreviations: MMP, matrix metalloproteinase; miRNAs, microRNAs; 3′-UTR, 3′-untranslated region; RECK, reversion-inducing cysteine-rich protein with Kazal motifs; ECM, extracellular matrix; CCTA, coronary computed tomographic angiography; CACS, coronary artery calcium score; MDM, monocyte-derived macrophages; qRT-PCR, quantitative reverse transcription polymerase chain reaction; PBMC, peripheral blood mononuclear cells; rhGM-CSF, recombinant human granulocyte macrophage colony-stimulating factor; cel-miR, *Caenorhabditis elegans* miR; ELISA, enzyme linked immunosorbent assay; Ct, threshold cycle; siRNA, small interfering RNA; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROC, receiver operating characteristic; CAD, coronary artery disease; VSMCs, vascular smooth muscle cells.

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protein translation, miRNAs have emerged as powerful regulators of various main biological processes, including development, differentiation, cell proliferation and apoptosis (Ambros, 2004; Carrington and Ambros, 2003; Hwang and Mendell, 2006).

miR-21 is involved in several crucial processes of atherosclerosis (Gabriely et al., 2008; Hashimi et al., 2009; Schmeier et al., 2009; Weber et al., 2010). Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a membrane-anchored MMP inhibitor and it has been reported as direct target genes of miR-21 (Han et al., 2012). Endogenous inhibitors of MMPs play a critical role in extracellular matrix (ECM) homeostasis which is important in malignancy invasion and metastasis (Alexander et al., 1996), and recent evidence also suggests that miR-21 could promote cancer invasion by targeting MMP regulators (Gabriely et al., 2008; Selaru et al., 2009). Although the process of matrix-degrading in coronary atherosclerotic unstable plaque resembles the malignancy invasion and metastasis, miR-21's impact on the human coronary atherosclerotic plaque instability has not been reported. Therefore in this study we try to evaluate the role of miR-21 in plaque instability by studying the pathway of miR-21 on MMPs and its inhibitor RECK in macrophages.

Materials and methods

Study population

Outpatients with complaints of chest pain were recruited in Fuwai Hospital (Beijing, China) between August 2011 and January 2013 (Table 1). All of them underwent coronary computed tomographic angiography (CCTA) by a 64-slice multi-detector CT scanner (LightSpeed VCT; GE Healthcare, Milwaukee, WI, USA). In this cohort, patients in non-calcified plaque group had typical non-calcified plaque [coronary artery calcium score (CACS) < 50 HU] without calcified and mixed plaque on the main coronary artery. Patients with typical calcified plaque (CACS > 100 HU) (no non-calcified and mixed plaque) were enrolled into calcified plaque group. Patients without any coronary plaque served as the control group. General exclusion criteria were factors that would impact the accurate measurement of biomarkers (fever, inflammatory diseases, acute myocardial injury, severe heart failure, malignant disease, impaired liver function, renal failure, or recent surgery).

Among them, 3 non-calcified plaque patients, 2 calcified plaque patients and 9 controls were recruited to draw and culture monocyte-derived macrophages (MDM) from peripheral blood for miR-21 expression testing. 63 non-calcified plaque patients, 62 calcified plaque patients and 61 controls were recruited for the serum miR-21 and MMP-9 detection. The ethics review board of the Fuwai Hospital approved the protocols, and written informed consent was obtained from each individual.

Generation and culture of human MDM

Isolation of peripheral blood mononuclear cells (PBMCs) and culture of MDM were performed using classical methods (van Grevenynghe et al., 2003; Young et al., 1990). The PBMCs were isolated for 1 h after blood collection. PBMCs were isolated through Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, NJ, USA) density gradient centrifugation. Then cells were plated in culture flasks with RPMI 1640 medium (Gibco, CA, USA) containing 20% fetal bovine serum (FBS) and penicillin/streptomycin (1000 U/mL each), at a density of 5 × 106 cells/mL, and incubated at 37 °C, 5% CO_2 for 3 h to adhesion. Adherent monocytes were next cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics in the presence of 1000 U/mL recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) (PeproTech, New Jersey, USA) to get macrophages. Experiments for MDM were performed after 6 days of the addition of rhGM-CSF.

RNA extraction

Total cell RNA was isolated from the cultured macrophages using TRIzol according to the manufacturer's instructions, and then was quantified by the NanoDrop 2000 spectrophotometer.

Total serum RNA was extracted with the mirVana PARIS Kit (Ambion, Warrington, United Kingdom) according to the manufacturer's protocol (Mitchell et al., 2008). Total RNA was purified from 400 μL of serum and eluted into 50 μL by RNase-free water. 5 nmol/L *Caenorhabditis elegans* (cel)-miR-39, cel-miR-54 and cel-miR-238 were spiked-in the samples (after addition of 2× Denaturing Solution) as housekeeping miRNA (as a mixture of 25 fmol of each oligonucleotide in a 5 μL total volume) (Kroh et al., 2010; Mitchell et al., 2008).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay

First, 5 μL RNA was put into 15 μL reverse transcription (RT) reaction system using the TaqMan miRNAs RT Kit (Part No. 4366597, Applied Biosystems, Foster City, USA) and miR-21-specific stem-loop primers (AB Assay ID 000397) to generate cDNA according to the manufacturer's protocol (Kroh et al., 2010). The following conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min and held at 4 °C, were used to carry out the RT reactions in Tetrad 2 Peltier Thermal Cycler (Bio-Rad, CA, USA). Subsequently, qRT-PCR was performed on ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, USA). The TaqMan 2× Universal PCR Master Mix (No AmpErase UNG) and TaqMan miRNA Assay (Applied Biosystems, Foster City, USA) were used for 10 min at 95 °C, followed by 15 s (40 cycles) at 95 °C and 60 °C for 1 min (40 cycles). The threshold cycle (Ct) value was defined as the cycle number at

Table 1
Characteristics of study populations.

Characteristics	Control group (n = 61)	Calcified plaque group (n = 62)	Non-calcified plaque group (n = 63)	p value
Age (yrs)	53.69 ± 8.82	57.73 ± 9.26	55.71 ± 9.47	0.06
Men (%)	28(45.90%)	33(53.23%)	35(58.73%)	0.36
Body mass index (kg/m^2)	25.22 ± 3.29	25.56 ± 3.42	26.35 ± 3.09	0.14
Laboratory profile				
Total cholesterol (mmol/L)	4.81 ± 0.93	4.83 ± 1.09	4.87 ± 1.14	0.93
LDL-cholesterol (mmol/L)	2.92 ± 0.79	2.91 ± 0.97	2.94 ± 0.97	0.99
HDL-cholesterol (mmol/L)	1.36 ± 0.35	1.35 ± 0.33	1.22 ± 0.35	<0.05
ApoA1 (g/L)	1.67 ± 0.37	1.68 ± 0.28	1.60 ± 0.30	0.30
ApoB (g/L)	1.06 ± 0.28	1.12 ± 0.34	1.21 ± 0.34	<0.05
Triglycerides (mmol/L)	1.44(0.99)	1.44(1.36)	1.71(1.39)	<0.05
C-reactive protein (mg/dL)	1.35(2.24)	1.44(2.49)	1.70(2.54)	0.12
Glucose (mmol/L)	5.66 ± 0.92	6.34 ± 2.13	6.18 ± 1.63	0.06

Data are presented as means (±SD) or number (%) or median (interquartile).