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MicroRNA Associated with Atherogenic Dyslipidemia in South Asian Men

Elena Flowers¹, Komal Singh², César Molina², Ashish Mathur², and Bradley E. Aouizerat^{1,3}

¹Department of Physiologic Nursing, School of Nursing, University of California, San Francisco

²South Asian Heart Center, El Camino Hospital

³Institute for Human Genetics, University of California, San Francisco

Keywords

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MicroRNA (miR) are structural components of an epigenetic mechanism of post-transcriptional regulation of mRNA. There is mounting evidence to support a role for miR in regulation of lipoprotein metabolism by influencing lipoprotein synthesis, reverse cholesterol transport, and insulin signaling. [1] The majority of studies to date have been performed in vitro or in animal models [1], and less is known about the impact of miR on lipoprotein metabolism in humans. South Asians are disproportionately afflicted with early, severe atherosclerotic cardiovascular disease. [2] Dyslipidemia is prevalent in 25% - 65% of the South Asian population, appears to be a primary contributor to disease [3], and is often characterized by normal levels of total cholesterol and LDL, but low HDL and elevated triglycerides [4]. The purposes of this study were to measure miR species known to be prevalent in human blood, to determine their expression in South Asian men with and without atherogenic dyslipidemia using array-based methods, and to validate expression of miR with plausible biologic function in atherogenic dyslipidemia via quantitative polymerase chain reaction (qPCR).

This was a nested case-control study of South Asian participants in a cardiovascular risk reduction program. Individuals taking cholesterol medication, with a body mass index <20kg/m² or >35kg/m², current smokers, heavy alcohol users, and those with previously diagnosed diabetes were excluded. Men with HDL < 40mg/dL and triglycerides > 150mg/dL were defined as cases. This study was approved by the Committee on Human Research at the University of California, San Francisco.

Blood for miR analysis was collected into PAXgene tubes (PreAnalytiX, Switzerland). The PAXgene Blood microRNA Kit (PreAnalytiX, Switzerland) was used to isolate miR from whole blood. For array analysis, pools of RNA representing cases (20 samples) and controls

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Corresponding Author: Elena Flowers, PhD Assistant Professor University of California, San Francisco Department of Physiological Nursing 2 Koret Way, #605L San Francisco, CA 94143-0610 Phone: 415-476-0983 Fax: 415-476-8899 eflowers@ucsf.edu.

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(20 samples) were created based on the miR concentration. Total RNA from individual samples and pools was reverse transcribed into complementary DNA (cDNA) using the miScript II RT Kit (Qiagen, Valencia, CA). Simultaneous detection of 85 miR and small nuclear RNA U6 (RNU6) was performed using the miScript microRNA PCR Array for human serum and plasma in 384 well plate format (Catalog # MIHS-106Z, Qiagen, Valencia, CA). Pooled sample measurements were performed in sextuplicate. Quantitative polymerase chain reaction (qPCR) was performed using the miScript SYBR Green PCR Kit (Qiagen, Valencia, CA).

Descriptive statistics and Student's t-test were performed to compare demographic and clinical characteristics between cases and controls. All statistical analyses were performed using Stata 11 (College Station, TX) and Microsoft Excel (Redmond, WA). MiR displaying at least 2-fold statistically significant ($p < 0.05$) differences in expression by both Wilcoxon test of equality and Student's t-test in the array-based experiment, which also target biologically relevant mRNA, were selected for qPCR validation.

Twenty-two cases and 22 controls were enrolled. The mean age was 42 ± 2 years (Table 1). Cases had lower HDL (33 mg/dL vs. 47 mg/dL, $p < 0.05$) and higher triglycerides (237 mg/dL vs. 109 mg/dL, $p < 0.05$) than controls. Cases had higher fasting blood glucose (97 mg/dL vs. 88 mg/dL, $p < 0.05$) and were overweight (body mass index 26.1 kg/m^2 vs. 23.9 kg/m^2 , $p < 0.05$), likely due to abdominal adiposity (waist circumference 94 cm vs. 82 cm, $p < 0.05$). In contrast, there were no statistically significant differences between cases and controls in total cholesterol (197 mg/dL vs. 194 mg/dL, $p = 0.8$) or LDL (116 mg/dL vs. 123 mg/dL, $p = 0.3$).

Of the 85 miR measured by array, 74 (87%) were detected with sufficient precision to meet all of the quality control criteria. Three miR (miR-214, miR-885-5p, miR-205) displayed increased expression in cases compared to controls. Fifteen miR (miR-374a, miR-100, miR-7, miR-18a, miR-125b, miR-148a, miR-17, miR-21, miR-221, miR-93, miR-143, miR-17*, miR-106b, miR-96, miR-20a) displayed at least two-fold decreased expression ($p < 0.05$) in cases compared to controls (Table 2). Six miR (miR-100, miR-106b, miR-125b, miR-148a, miR-21, miR-7) previously shown to regulate the expression of gene pathways that regulate lipoprotein metabolism were selected for further analysis by qPCR. The only miR that remained statistically significant when measuring individual samples was miR-21 ($p < 0.05$) (Table 2).

To our knowledge, this is the first study to examine circulating levels of candidate miR in humans with atherogenic dyslipidemia. MiR appear to have regulatory functions in lipoprotein metabolism, however little is known about the possibility of using miR as a blood-based biomarker for atherogenic dyslipidemia. We found that 15 miR species exhibit at least two-fold statistically significant differential expression in cases compared to controls. Of these, six with *a priori* evidence for a role in lipid metabolism and related physiological conditions (i.e., inflammation, atherosclerosis, insulin resistance) were validated by qPCR and one (miR-21) was found to be differentially expressed ($p < 0.05$). Although the agreement between the array-based measurement and qPCR validation in both pooled and individual samples for the other five targets (miR-100, miR-106b, miR-125b, miR-148a, miR-7) was high, they failed to achieve statistical significance in the qPCR validation experiments.

MiR-21 is positively associated with hypertrophy (i.e., heart failure) [5] and ischemic vascular disease, [6] and over time, this proliferative effect of miR-21 may become maladaptive [7]. Increased hepatic miR-21 is associated with decreased insulin resistance [8] that is likely to culminate in anti-atherogenic changes in lipoprotein profiles [9]. Given the

fate of miR intracellularly, [1] one could hypothesize that increased demand for miR-21-related regulation of gene expression intracellularly would result in decreased excretion of miR-21 into the circulation. Although this has not been demonstrated specifically for miR-21, it has been demonstrated for two other miR (i.e., miR-17-5p, miR-132). [10] Thus, the observed decreased levels of miR-21 in men with atherogenic dyslipidemia as compared to controls may result from decreased excretion of intracellular miR-21. Further research is warranted to determine if intracellular miR-21 is inversely correlated with circulating miR-21 and if intracellular miR-21 represents a physiological attempt to mitigate atherogenic dyslipidemia or becomes maladaptive in this context. Such work could shed light on the utility of circulating miR-21 as a prognostic or diagnostic marker of disease.

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Table 1

Clinical Characteristics of Participants

Characteristics Mean $\pm$ SD or n (%)	Cases (n = 22)	Controls (n = 22)	p-value
Age (years)	43 $\pm$ 2	43 $\pm$ 2	0.84
Systolic Blood Pressure (mm Hg)	129 $\pm$ 16	116 $\pm$ 29	0.06
Diastolic Blood Pressure (mm Hg)	80 $\pm$ 10	72 $\pm$ 20	0.08
Total cholesterol (mg/dL)	197 $\pm$ 28	194 $\pm$ 32	0.81
LDL (mg/dL)	116 $\pm$ 25	123 $\pm$ 30	0.26
HDL (md/dL)	33 $\pm$ 3	47 $\pm$ 7	<0.05
Triglycerides (mg/dL)	237 $\pm$ 70	109 $\pm$ 28	<0.05
Blood glucose (mg/dL)	97 $\pm$ 11	88 $\pm$ 9	<0.05
Waist circumference (cm)	94 $\pm$ 8	82 $\pm$ 19	<0.05
Body mass index (kg/m ²)	26.1 $\pm$ 3.1	23.9 $\pm$ 2.8	<0.05

Table 2

Fold change microRNA expression in men with compared to without atherogenic dyslipidemia

microRNA	Array-based measurement			qPCR validation			
	Fold change *	p-value ¥	p-value €	Individual samples (n=40)		Pooled samples	
				Fold change **	p-value ¥	Fold change **	p-value €
374a	-2.98	0.0104	0.0060				
100	-2.92	0.0039	0.0015	-2.07	0.3	-2.5	0.04
7	-2.78	0.0039	0.0004	-2.72	0.5	-1.51	0.02
18a	-2.78	0.0065	0.0034				
125b	-2.53	0.0039	0.0009	-1.78	0.1	-2.42	0.01
148a	-2.45	0.0039	0.0004	1.55	0.5	-2.15	0.02
17	-2.27	0.0104	0.0107				
21	-2.24	0.0039	0.0025	-1.88	0.03	-1.68	0.004
221	-2.23	0.0065	0.0075				
93	-2.17	0.0039	0.0009				
143	-2.06	0.0250	0.0106				
17 *	-2.05	0.0039	0.0037				
106b	-2.03	0.0104	0.0089	-1.63	0.3	-1.46	0.002
96	-2.03	0.0250	0.0223				
20a	-2.00	0.0039	0.0151				

* Fold change calculated using the following formula: $2^{-\frac{Ct_{\text{target}} - C_{\text{t}}}{C_{\text{t}}}}$ where $C_{\text{t}} = \text{mean} (Ct_{\text{target, cases}} - \text{mean} (Ct_{\text{normalizer, cases}})) - \text{mean} (Ct_{\text{target, controls}} - \text{mean} (Ct_{\text{normalizer, controls}}))$

** Fold change calculated using the following formula: $(E_{\text{target}} - Ct_{\text{target}}) / (E_{\text{normalizer}} - Ct_{\text{normalizer}})$ where $C_{\text{t}} = \text{mean} Ct_{\text{cases}} - \text{mean} Ct_{\text{controls}}$ using the mean of replicates for each sample after excluding outliers. Outliers were defined as greater than 0.5 standard deviations from mean C_{t} .

¥ p-value calculated using Wilcoxon non-parametric test of equality for unmatched pairs

€ p-value calculated using Student's t-test with unequal variance assumption