Circulating MicroRNA-423-3p Improves the Prediction of Coronary Artery Disease in a General Population

— Six-Year Follow-up Results From the China-Cardiovascular Disease Study —

Xin Wang, MD; Ying Dong, PhD; Tian Fang, BSc; Xiaoxia Wang, BSc;
Lu Chen, PhD; Congyi Zheng, PhD; Yuting Kang, PhD; Linlin Jiang, BSc;
Xin You, MD; Shujie Gai, BSc; Zengwu Wang, MD, PhD; Huiqing Cao, MD, PhD

Background: Circulating microRNAs (miRNA) are potential prognostic biomarkers for cardiovascular disease. We aimed to identify serum miRNA as an effective predictor for coronary artery disease (CAD) events in a general population cohort.

Methods and Results: Serum miRNAs associated with CAD were determined by small RNA sequencing and quantitative RT-PCR. Further, the predictive ability of identified serum miRNAs was measured in a general population of 2,812 people. As a main outcome measure, CAD events were collected for 6 years and included acute myocardial infarction and subsequent myocardial infarction. Out of the 48 miRNA candidates, 5 miRNAs (miR-10a-5p, miR-126-3p, miR-210-3p, miR-423-3p and miR-92a-3p) showed better reliability and repeatability in serum. Then, the association of serum levels of the 5 miRNAs with CAD was validated. Furthermore, miR-10a-5p and miR-423-3p, which showed better performance, were tested in the large cohort, with a median follow up of 6.0 years. In multivariable Cox regression analysis, only miR-423-3p (P for trend < 0.001) was able to precisely predict CAD events. Moreover, the addition of circulating miR-423-3p with the traditional risk factors together markedly improved the various model performance measures, including the area under the operating characteristics curve (0.782 vs. 0.806), Akaike Information Criterion (965.845 vs. 943.113) and net reclassification improvement (19.18%).

Conclusions: Circulating miR-423-3p can improve the prediction of primary CAD outcomes on the basis of a traditional risk factor model in general population.

Key Words: Biomarker; Circulating microRNA; Coronary artery disease; General population; Prediction

Cardiovascular disease (CVD) is one of the most important public health issues in the world.¹ Data from World Health Organization indicates that approximately 17.5 million people died from CVDs in 2012 (7.4 million caused by coronary artery disease (CAD)), which accounts for 31% of all global deaths.² Among CVDs, CAD remains the leading cause of mortality worldwide.^{3–5} In Asia, there is an increasing CAD burden because of rising prevalence of sedentary lifestyle and changes in food consumption.⁶ Early prevention of CAD is of clinical

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significance for the overall reduction of CVD mortality.⁷ This calls for biomarkers of CAD in clinical practice.

MicroRNAs (miRNAs) are small non-coding molecules that play an important role in development of atherosclerosis and their expression altered during CAD events.^{8,9} In 2008, Lawrie et al first reported the presence of miRNAs in biological fluids, which triggered an upsurge in research

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Division of Prevention Community Health, National Center for Cardiovascular Disease, Fuwai Hospital, Peking Union Medical College & Chinese Academy of Medical Sciences, Beijing (Xin W., L.C., C.Z., Y.K., L.J., Z.W.); Heart Center and Beijing Key Laboratory of Hypertension, Chaoyang Hospital, Capital Medical University, Beijing (Y.D.); Institute of Molecular Medicine, Peking University, Beijing (T.F., Xiaoxia W., H.C.); Department of Laboratory Medicine, The Affiliated Hospital of Yanbian University, Jilin (X.Y.); and State Key of Laboratory of Cardiovascular Disease, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing (S.G.), China

The first four authors contributed equally to this work (Xin W., Y.D., T.F., Xiaoxia W.).

Mailing address: Zengwu Wang, MD, PhD, Division of Prevention and Community Health, National Center for Cardiovascular Disease, Fuwai Hospital, Peking Union Medical College & Chinese Academy of Medical Sciences, No. 15 (Lin), Fengcunxili, Mentougou District, Beijing 102308, China. E-mail: wangzengwu@foxmail.com

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of circulating miRNAs as biomarker of various human disorders including CAD in the following decades.^{10–13} Ever-increasing studies demonstrated that circulating miRNAs are suitable biomarkers due to their specificity, stability, as well as easy availability in body fluids.^{14,15} Previously, several studies showed that circulating miRNA seem to be promising biomarker for the diagnosis of CAD.¹¹ However, limited information has been provided regarding the correlation of miRNAs with CAD prognosis and the predictive ability compared with previously established CAD biomarkers (such as high-sensitivity C-reactive protein (hs-CRP), high-sensitivity troponin, etc.).^{16,17} Thus, whether circulating miRNAs can serve as an effective predictive biomarker for CVD events in general populations is still unknown.

The aim of the present study was to assess whether miRNA can predict CAD in a primary prevention cohort of the China CVD risk factor study (the China-CVD study). With CAD events being recorded for 6 years, the large general population allows the evaluation of serum miRNA for CAD prediction. In particular, we posed the question of whether miRNA can improve the prediction of CAD beyond classical cardiovascular risk factors.

Methods

Study Design

The study design is shown in **Supplementary Figure 1**. Two steps were included to determine potential predictive miRNAs biomarkers in a large population. First, we aimed to screen miRNA candidates to distinguish CAD patients from healthy controls with good repeatability of polymerase chain reaction (PCR) detection from serum. For this purpose, differentially expressed miRNAs from the serum of CAD patients and controls were identified by small RNA sequencing. To improve the specificity for screening, we also sequenced miRNAs in peripheral blood monocytes (PBMC) of the CAD/control participants and in hypoxia/control-treated vascular endothelial cells (ECs). Potential miRNA candidates were listed according to their fold change and abundance in serum sequencing data. The miRNAs that have been dysregulated both in serum and PBMC or ECs were given priority for selection. The reliability and reproducibility of the PCR detection in serum for these miRNA candidates were further determined by PCR product sequencing and quantitative experiments. Next, the qualified candidates were measured in sporadic CAD populations to further confirm their abilities of distinguishing patients from controls. Second, the most reproducible miRNA candidates associated with CAD were chosen to determine their predictive effects on the risk of CAD in a large-scale general population.

Case-Control Study

The CAD patients and healthy volunteers for case-control analysis in this study were recruited from three hospitals in northern China. We used 2 populations; 1 population including 39 patients and 39 controls was recruited from Xuanwu Hospital, Capital Medical University and Affiliated Hospital of Jining Medical University; the other population for validation including 30 CAD patients and 21 controls was from Affiliated Hospital of Yanbian University. Clinical definition of CAD was confirmed by 2 experienced cardiologists, and CAD was defined if more than 50% diameter stenosis in at least 1 of the 3 major coronary arteries was shown by angiography. Control

individuals were recruited from health examination center of the hospitals according to criteria described previously.¹⁸

Cohort Subjects

The study cohort consisted of participants who participated in the China-CVD study from 2009–2010. It consists of 12 study sites, representing the southern and northern provinces, as well as the urban and rural population in China to reflect geographic distribution and socioeconomic development. Detailed methods used in the survey have been described previously.¹⁹ For the present study, 4 out of 12 study fields (Beijing, Haerbin, Hanzhong and Yuxian) completed the follow-up measurements until 2016–2017. Subjects with missing miRNAs measurements at baseline, or pre-existing CVD, were excluded.

Ethics Statement

The protocols of this study were carried out according to the principles of the Declaration of Helsinki and approved by the Medical Ethics Committee from Fuwai Hospital (Reference number: 2014534). Written informed consent was obtained from all participants prior to enrollment. The de-identified data will be made available to the public.

Laboratory Methods

Blood was drawn from patients who were in a standard fasting state and stored at 80°C until analysis. PBMC were extracted from the same whole blood sample using Ficoll, followed by gradient centrifugation. Serum glucose was detected by the glucose oxidase peroxidase method; concentrations of total cholesterol (TC) were measured by the glucose oxidase-polymerization method; high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured by the synthetic polymer/detergent method; hs-CRP was measured by the latex agglutination immunoturbidimetric assay. All biochemical indicators were analyzed using a Hitachi HITACHI17080 (Hitachi, Ltd., Tokyo, Japan) automatic biochemistry analyzer in China Isotope & Radiation Corporation/Beijing CIC Clinical Laboratory, Beijing, China.

Risk Factor Definition

Current smokers were defined as participants who have smoked at least 20 packages of cigarettes or 0.5 kg of leaf tobacco in their lifetime and are smoking now. Hypertension was defined as a systolic blood pressure ≥ 140 mmHg, and/or a diastolic blood pressure ≥ 90 mmHg, and/or use of antihypertensive medications within 2 weeks;²⁰ diabetes mellitus as a self-reported history of diabetes plus a fasting blood glucose level ≥ 7.0 mmol/L.²¹

RNA Isolation and Small RNA Sequencing

Total RNA from serum samples was extracted using TRIzol-LS and total RNAs from cells using TRIzol reagent (Sigma-Aldrich, Shanghai, China). The RNA of pooled serum / PBMC samples from 10 CAD patients and 10 healthy controls were isolated and quantified for small RNA sequencing. Small RNA library preparation and sequencing were performed at sequencing Center of Peking University using the Illumina HiSeq2500 platform. The depth of sequencing was 4G.

We used the microRNAcute (Transgene, Beijing, China) to isolate miRNA from serum. Cel-miR-39 was used as spike-in control to monitor extraction efficiency. MiRNA in 200 μ L serum from each sample was extracted according

Figure. Serum levels and distinguishing ability of miR-10a-5p and miR-423-3p in coronary artery disease patients. The box plots showed the serum levels of miR-10a-5p (**A**) and miR-423-3p (**C**) in CAD patients, and miR-423-3p (**E**) in validated CAD patients by quantitative polymerase chain reaction. The receiver operating characteristic curve of serum miR-10a-5p (**B**) and miR-423-3p (**D**) in CAD patients, and serum miR-423-3p (**F**) in validated CAD patients. CAD, coronary artery disease; HC, health control.

to the procedure required by the kit.

Reverse Transcription and Quantitative Polymerase Chain Reaction

500 ng of RNAs isolated from serum was reverse-transcribed in a final volume of 20 μ L using a reverse transcription kit (Transgene) with miRNA-specific stem-loop RT primers (RiboBio, Guangzhou, China). MiRNA was amplified using miRNA-specific detection primers (RiboBio) and quantified using SYBR green dye-based quantitative PCR (RiboBio). UniSp6 spike-in control (Exiqon, Denmark) was used to confirm the equal efficiency of cDNA-synthesis performed in different samples. PCR products were

inserted into the pGM-T vector for miRNA sequencing. For fold change analysis, the $2^{-\Delta\Delta C_t}$ method was used to calculate miRNA levels. For miRNA normalization in the small-scale CAD case-control populations, the universally accepted small RNA internal control, miR-16 and U6, were tested, and U6 was finally used due to its good stability in different groups. In the large China-CVD populations, overall average was used for circulating miRNA normalization, according to NormFinder analyses. The miRNA data were normalized to the average number of assays detected to obtain a ΔC_t value.

Table 1. Baseline Characteristics of Participants in the China-Cardiovascular Disease Population Cohort

	Total	miR-10-5p				miR-423-3p			
		Tertile 1	Tertile 2	Tertile 3	P for trend	Tertile 1	Tertile 2	Tertile 3	P for trend
n, %	2,812 (100.0)	934 (33.2)	936 (33.3)	942 (33.5)		936 (33.3)	940 (33.4)	936 (33.3)	
Age (years)	51.2±7.9	51.0±7.9	51.2±7.9	51.5±7.9	0.791	51.2±7.9	51.3±7.8	51.1±8.1	0.780
Male	1,197 (42.6)	372 (39.8)	398 (42.5)	427 (45.3)	0.008	413 (44.1)	380 (40.4)	404 (43.2)	0.337
BMI (kg/m ²)	25.2±3.6	25.3±3.4	25.6±3.8	24.7±3.6	<0.001	25.4±3.6	25.5±3.5	24.6±3.6	<0.001
Smoking status	796 (28.3)	261 (27.9)	267 (28.5)	268 (28.5)	0.404	270 (28.9)	248 (26.4)	278 (29.7)	0.341
Alcohol consumption	347 (12.3)	99 (10.6)	118 (12.6)	130 (13.8)	0.018	115 (12.3)	102 (10.9)	130 (13.9)	0.146
SBP (mmHg)	135.0±20.3	135.2±19.9	134.8±20.0	135.1±21.0	0.873	135.6±20.6	134.4±19.5	135.0±20.7	0.680
DBP (mmHg)	84.1±11.1	83.8±10.8	84.3±11.3	84.3±11.2	0.545	84.3±11.1	84.2±10.5	83.9±11.6	0.966
FBG (mmol/L)	6.05±2.11	5.95±1.80	6.12±2.27	6.07±2.23	0.829	6.14±2.38	6.01±1.87	5.99±2.05	0.223
TC (mmol/L)	4.74±0.94	4.58±0.90	4.76±0.95	4.87±0.96	<0.001	4.71±0.96	4.78±0.96	4.73±0.91	0.916
HDL-C (mmol/L)	1.33±0.29	1.31±0.29	1.32±0.29	1.36±0.30	<0.001	1.32±0.30	1.32±0.28	1.36±0.30	0.002
LDL-C (mmol/L)	2.69±0.81	2.57±0.75	2.71±0.81	2.79±0.84	<0.001	2.67±0.83	2.74±0.82	2.67±0.77	0.549
hs-CRP (mg/L)	1.84±3.14	1.77±3.03	1.90±3.19	1.83±3.20	0.661	1.89±3.26	1.82±2.77	1.79±3.37	0.418
Hypertension	1,315 (46.8)	419 (44.9)	428 (45.7)	468 (49.7)	0.018	438 (46.8)	437 (46.5)	440 (47.0)	0.463
Diabetes mellitus	395 (14.1)	117 (12.5)	141 (15.1)	137 (14.5)	0.105	136 (14.5)	122 (13.0)	137 (14.6)	0.474
Statin therapy	58 (2.1)	27 (2.9)	18 (1.9)	13 (1.4)	0.011	21 (2.2)	20 (2.1)	17 (1.8)	0.258
Antihypertensive therapy	461 (16.4)	172 (18.4)	138 (14.7)	151 (16.0)	0.082	164 (17.5)	182 (19.4)	115 (12.3)	0.001

Data are presented as mean±standard deviation and n (%). BMI, body mass index; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol.

Cell Culture and Treatment

Human umbilical vascular ECs (HUVECs) were cultured in EC medium (ScienCell, USA), supplemented with 5% fetal bovine serum, 1% EC growth supplement and 1% penicillin/streptomycin. HUVECs within 3–7 passages were used in this study. For hypoxia treatment, cells were incubated at 37°C under 1% O₂, 5% CO₂ and 94% N₂ for different time. Tumor necrosis factor alpha (TNF-α) (Peprotech, USA) was used in the experiments, with a final concentration of 20 ng/mL. Exosomes were isolated from harvested culture medium, as previously described.²² Culture medium were collected on ice and centrifuged to remove cells and cellular debris, then supernatants were filtered and concentrated using an Amicon Ultra filter (Millipore, Billerica, MA), following the manufacturer's instructions. Exosomal RNA isolation was performed using microRNAacute (Transgene).

End-Point Ascertainment

The primary endings were CAD events, which included acute myocardial infarction (ICD-10 pos. I21) and subsequent myocardial infarction (ICD-10 pos. I22).²³ All events were identified and reported by trained researchers and physicians based on medical records. Furthermore, all outcomes were adjudicated by the independent committee according to the standardized definitions. Only the first event was used for analysis.

Statistical Analysis

Continuous variables were presented as means±standard deviations (SDs), and categorical variables as number (proportions). One-way ANOVA and a Chi-squared test were used to compare the characteristics of study participants at baseline by tertiles of miR-10a-5p and miR-423-3p. Cox proportional hazard models were used to assess the association of miR-10a-5p and miR-423-3p with CAD

events. When modeling, the values of these 2 miRNA candidates were used as both continuous variables and categorical variables (three categories, grouped by tertile, where the lowest tertile was used as the reference). Model 1 was adjusted for age and gender; model 2 further adjusted for smoking status, hypertension, diabetes mellitus, body mass index (BMI), HDL-C and LDL-C. The discriminatory ability and model fit were compared between the base prediction model (model A: according to classical risk factors: age, gender, smoking status, hypertension, diabetes mellitus, BMI, HDL-C and LDL-C) and new prediction model (model B: model A+miRNA candidates). Model discriminatory was assessed using the area under the operating characteristics curve (AUC); model fit was assessed using the Akaike Information Criterion (AIC). Furthermore, net reclassification improvement (NRI) was also calculated to test whether addition of miRNA candidates can improve the risk classification. Finally, a comparison between the hs-CRP prediction model (model C: classical risk factors+hs-CRP) and new prediction model (model B) was made using AUC and AIC to address whether the miRNA candidates had incremental predictive utility over hs-CRP. The NRI was analyzed using the Z test.^{24,25} The AUC was calculated using R software (version 3.4.4; <http://www.r-project.org>). Other statistical analysis was performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). A two-sided P<0.05 was considered statistically significant.

Results

Identification of miRNA Candidates Associated With CAD

To screen CAD-associated miRNA candidates effectively and systematically, genemo-wide miRNAs were sequenced from serum of CAD patients/healthy controls. 1,022 miRNAs in patients and 976 miRNAs in controls were detected, and

Table 2. Risk of CAD According to Baseline miRNA Candidate Tertiles

	Person-years	No. of events	Morbidity (1,000-person rate)	Model 1	Model 2
miR-10a-5p					
T1	5,519.4	16	2.9	1.00 (Ref.)	1.00 (Ref.)
T2	5,529.2	30	5.4	1.84 (1.00–3.37)	1.70 (0.92–3.14)
T3	5,619.1	18	3.2	1.06 (0.54–2.08)	1.05 (0.53–2.09)
P for trend				0.470	0.341
miR-423-3p					
T1	5,459.2	36	6.6	1.00 (Ref.)	1.00 (Ref.)
T2	5,599.2	18	3.2	0.49 (0.28–0.87)	0.48 (0.27–0.84)
T3	5,609.3	10	1.8	0.27 (0.13–0.54)	0.28 (0.14–0.57)
P for trend				<0.001	<0.001

Model 1: Adjustment for age, sex. Model 2: Model 1 plus adjustment for smoking status, body mass index, hypertension, diabetes mellitus, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol. CAD, coronary artery disease; miR, microRNA; T1–3, tertile upper, middle, lower.

differentially expressed miRNAs were listed according to their fold changes and abundance. 129 miRNAs (fold change ≥ 1.5 or ≤ 0.7 , reads per kilobase per million (RPKM) > 50) were selected as the first candidates (**Supplementary File**). Then, to further narrow the scope of candidates from the 129 miRNAs, we selected 48 miRNAs according to the following criteria: (1) dysregulated in sequencing data of hypoxic ECs (FC ≥ 1.2 or ≤ 0.8 , RPKM > 20); and (2) miRNAs were also changed in PBMCs of CAD patients or in hypoxic endothelial cells. Finally, 48 miRNAs were selected for further qPCR detection in serum (**Supplementary Table 1**). We found that 5 miRNAs (miR-10a-5p, miR-126-3p, miR-210-3p, miR-423-3p and miR-92a-3p) could be specifically and stably detected in serum by PCR product sequencing (**Supplementary Figure 2**).

MiR-423-3p was the newly identified candidate in CAD, whereas the other 4 miRNAs have been previously documented as CAD biomarkers.^{11,26} To further confirm the association of the 5 miRNAs with CAD, we then measured their serum levels in 39 CAD patients and 39 healthy controls (**Supplementary Table 2**). Our data showed that serum levels of all measured miRNAs, with the exception of miR-92a-3p, were significantly lower in CAD patients compared with healthy controls (**Supplementary Table 3**). We demonstrated the fold change of miR-10a-5p and miR-423-3p in CAD patients (**Figure A,C**) and analyzed their abilities to distinguish CAD from controls by the AUC (**Figure B,D**).

To further validate the association of miR-423-3p with CAD, we measured serum miR-423-3p in another 30 CAD patients and 21 controls (**Supplementary Table 4**). It was consistently found that the level of miR-423-3p in CAD was significantly lower than that in controls, with a good distinguishing ability (AUC=0.8) (**Figure E,F**).

To explore the relationship between 4 miRNA candidates and pathology of the CAD process, we determined the expression of miR-423-3p in ECs treated with hypoxia and inflammatory cytokine TNF- α . We found miR-423-3p was markedly downregulated by TNF- α , while unaffected by hypoxia (**Supplementary Figure 3A,B**). To confirm the mechanism for miR-423-3p to be released from ECs, we performed an experiment by isolating exosome from medium of TNF- α treated/untreated ECs; miR-423-3p levels in the exosome were measured by using qPCR. The results showed decreased miR-423-3p levels in the extra-

cellular exosomes, which is consistent with that in ECs (**Supplementary Figure 3C**).

Association Between Serum Levels of miRNAs and CAD Events in a General Population

Table 1 shows the baseline characteristics of the China-CVD cohort population by tertiles of miR-10a-5p and miR-423-3p (n=2,812, mean age: 51.2 years, male: 42.6%). After a median duration of 6.0 years follow up, 64 CAD events, 68 stroke events, and 76 deaths from all-cause mortality occurred. Of death events, 13 participants died from stroke events, 0 from CAD events. In order to assess the association of miRNA candidates with CAD, analyses adjusted for age, gender and other classical cardiovascular risk factors, were performed (**Table 2**). We found that miR-423-3p level was negatively correlated with CAD risk (P for trend<0.001), but miR-10a-5p did not show an association with CAD risk.

Prediction of CAD by Serum Levels of miRNA Candidates

Supplementary Table 5 shows the AUC and AIC for CAD events by different prediction models. First, after adding miR-10a-5p to the base prediction model, the AUC and AIC values remained mainly unchanged. However, the model discrimination, due to the addition of miR-423-3p, was improved significantly, with the AUC increased from 0.782 to 0.806 for CAD (P=0.046). Likewise, great AIC differences were observed in the models with and without miR-423-3p (943.113 vs. 965.845 for CAD). **Tables 3** and **4** show the NRI by adding miR-10a-5p and miR-423-3p into the base model of CVD risk factors, respectively. However, we only found that the addition of miR-423p could improve the NRI. A total of 19 (5+2+12) individuals who developed CAD were reclassified upward, and 6 (1+0+5) participants who developed CAD were reclassified downward. The net estimate for who developed CAD events was the difference between these 2 estimates divided by the total number of CAD events $[(19-6)/64=20.31\%]$. Likewise, the net estimate for those participants without CAD events was -1.11% $[(134+3+52)-[122+28+70]]/2,748=-1.11\%$. Thus, the NRI was 19.18% (P=0.002).

Finally, we also made a comparison of predictive ability of CAD between hs-CRP and miRNA candidates (**Supplementary Table 6**). Cox regression analyses adjusted for age, gender and other cardiovascular risk factors showed

Table 3. NRI by Adding miR-10a-5p to the Risk Factors Independently Associated With CAD							
Predicted risk (with miR-10a-5p)	Reclassified predicted risk (with miR-10a-5p)						Total
	0–5%		5–10%		≥10%		
	n	%	n	%	n	%	
With CAD (n=64)							
0–5%	20	100.00	1	4.35	0	0.00	21
5–10%	0	0.00	22	95.65	1	4.76	23
≥10%	0	0.00	0	0.00	18	95.24	20
Total	20		23		21		
Without CAD (n=2,748)							
0–5%	2,041	98.17	36	8.20	0	0.00	2,077
5–10%	38	1.83	391	89.07	20	8.70	449
≥10%	0	0.00	12	2.73	210	91.30	222
Total	2,079		439		230		

CAD, coronary artery disease; miR, microRNA; NRI, net reclassification improvement. Estimates of probabilities using classical risk factors (vertical axis) and classical risk factors with miR-10a-5p (horizontal axis) are shown. NRI=2.91% (P=0.188).

Table 4. NRI by Adding miR-423-3p to the Risk Factors Independently Associated With CAD							
Predicted risk (with miR-423-3p)	Reclassified predicted risk (with miR-423-3p)						Total
	0–5%		5–10%		≥10%		
	n	%	n	%	n	%	
With CAD (n=64)							
0–5%	14	93.33	5	25.00	2	6.90	21
5–10%	1	6.67	10	50.00	12	41.38	23
≥10%	0	0.00	5	25.00	15	51.72	20
Total	15		20		29		
Without CAD (n=2,748)							
0–5%	1,927	93.36	122	29.12	28	10.57	2,077
5–10%	134	6.49	245	58.47	70	26.42	449
≥10%	3	0.15	52	12.41	167	63.02	222
Total	2,064		419		265		

Abbreviations as in Table 3. Estimates of probabilities using classical risk factors (vertical axis) and classical risk factors with miR-423-3p (horizontal axis) are shown. NRI=19.18%, P=0.002.

that, with the exception of hs-CRP (hazard ratio (HR) 1.13 per 1 SD increase, P=0.184) and miR-10a-5p (HR 1.14 per 1 SD increase, P=0.34), only miR-423-3p (HR 0.59 per 1 SD increase, P<0.001) reliably predicted CAD in the general population. Moreover, the miR-423-3p remained significantly associated with CAD events (HR 0.58 per 1 SD increase, P<0.001), after adjusting for traditional cardiovascular risk factors and hs-CRP; in contrast, the hs-CRP was not significant (HR 1.16 per 1 SD increase, P=0.108) after adjustment for traditional cardiovascular risk factors and the miR-423-3p. In addition, the AUC also showed excellent value with the addition of miR-423-3p into model A of CAD compared with the base prediction model+hs-CRP (P=0.047).

Discussion

In this study, we evaluated the predictive role of circulating miRNAs for CAD. Through a systematic and rigorous screening process, miR-423-3p was found effective for new CAD prediction in a general Chinese population. We found that higher circulating level of miR-423-3p was significantly associated with the lower risk of CAD in the prospective

population-based cohort. Importantly, the addition of miR-423-3p to the traditional risk factors significantly improved the ability of discrimination and reclassification of CAD events; its predictive performance is better than hs-CRP. We thereby have provided evidence that circulating miR-423-3p could serve as a novel biomarker in CAD prediction.

In 2008, the presence of miRNAs in biological fluids was first reported,¹³ which triggered an upsurge in research of circulating miRNAs as biomarkers of various human disorders including CAD in following decades.^{10–12,14,15} In contrast to the numerous studies in CAD diagnosis, the predictive value of miRNAs in a general population is less known. Although there is a great demand for new biomarkers for prediction and prevention of the high incidence disease, lacking of reliable miRNA candidates and large-scale cohort studies are the major limitations on the clinical application of the biomarkers.

In our study, based on small RNA sequencing, followed by a systematic and rigorous selection process, 5 miRNAs with good specificity and reproducibility were found suitable for PCR-based measurement in a large population. We speculated the stability and reproducibility of the

miRNA candidates may be closely associated with their abundance, existence form in serum, and sensitivity to PCR detection. We assessed the predictive value of 2 miRNAs in general population and identified miR-423-3p as an effective risk factor. Previous studies have identified individual or combined circulating miRNAs as novel biomarkers for prognosis of post-ACS, myocardial infarction or cardiovascular death in CAD patients.^{17,26,27} A report suggested a panel of circulating miRNAs improved the predictive power of Framingham Risk Score for acute myocardial infarction.²⁸ These studies were conducted either in patients with disease or in a small-scale population. Here, we have provided evidence that circulating miRNA could serve as an additional predictor for CAD occurrence in a large general population.

miR-423-3p, previously considered as an oncogene in several types of cancers, was also identified as a biomarker for lung cancer.²⁹ In this study, we found miR-423-3p was a responder for inflammatory factor of TNF- α , suggesting a possible role of the miRNA in EC activation, the critical step of atherosclerosis. Most circulating miRNAs were from multiple sources. Recent reports indicated that miR-423-3p in myocardial cells or cardiac fibroblasts were downregulated by ischemia-reperfusion injury.^{30,31} Here, the main sources of serum miR-423-3p in vivo could not be determined by experiments, and needs to be investigated in further studies.

The present study showed that miR-423-3p levels are associated with the risk of CAD events in a general population. Together with the traditional risk factors, circulating miR-423-3p can significantly improve the primary outcomes prediction. These results suggested that the circulating miR-423-3p plays an important role in the prediction of arteriosclerosis-related diseases. Meanwhile, our study also had several limitations that needed to be addressed. First, expanding validation studies into larger geographically independent cohorts is necessary to test fully the utility of miR-423-3p. In addition, we only measured hs-CRP at baseline, and the results show the predictive ability of miR-423-3p to detect CAD is better than hs-CRP; other established predictive biomarkers of CAD, such as high-sensitivity troponin,¹⁷ were not included in this study. Finally, because of the low number of CAD events, the results should be interpreted with caution, due to a possible limited power.

In summary, this is the largest study so far evaluating the prognostic value of circulating miRNAs in a general population in China. And it shows that the miR-423-3p derived from serum could significantly improve the prediction of CAD based on classical cardiovascular risk factors, and thereby could be a valuable biomarker for risk assessment in CAD.

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Disclosures

The authors declare that there are no conflicts of interest.

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Supplementary Files

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