Serum microRNA-204 Levels are Associated with Long-Term Cardiovascular Diseases Risk Using Framingham Risk Score in Patients with Type 2 Diabetes: Results From an Observational Study

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Research article

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Abstract

Background: The basic studies have demonstrated that microRNA-204 (miRNA-204) was involved in the process of atherosclerosis and vascular calcification. However, the value of miRNA-204 as a predictive biomarker for cardiovascular disease (CVD) is still controversial. The purpose of the present study was to evaluate the association between circulating miRNA-204 level and the 10-year cardiovascular disease risk score, Framingham risk score (FRS).

Method: The subjects consecutively enrolled 194 patients with type 2 diabetes mellitus without cardiovascular disease at Anzhen Hospital from January 2015 to September 2016. We used the Framingham Risk Score (FRS) to evaluate the risk of cardiovascular disease. Circulating miRNA-204 levels were measured by quantitative Real-Time polymerase chain reaction (qRT-PCR).

Result: The circulating miRNA-204 levels were significantly lower in high risk group of CVD (FRS > 20%) of patients (0.49 ± 0.13) compared with that in low risk group (FRS < 10%) and intermediate risk group (FRS = 10%-20%) (0.87 ± 0.19, 0.75 ± 0.25, Respectively, p < 0.001). FRS was negatively correlated with miR-204 levels (r=-0.421, p < 0.001). According to multivariate logistic analyses, miRNA-204 levels were still significantly and independently associated with the high risk of CVD after adjusting the conventional risk factor. Receiver-operating characteristic curve (ROC) analysis also showed that circulating miRNA-204 level can predict the high risk of CVD, and the specificity was higher than traditional risk factors SBP and protective factor HDL-C of CVD.

Conclusion: Our study demonstrated that patients with lower circulating miRNA-204 levels were at a high risk for the progression of CVD. After adjustment for potential confounders, miRNA-204 was independently associated with CVD in patients with T2DM.

Background

Previous investigations have shown that miRNAs played important roles in physiological and pathological processes, including cell cycle regulation[1], cell differentiation[1] inflammatory responses[2], apoptosis[3] and extracellular matrix (ECM) degradation[4]. Upgraded serum miRNA-204 (miR-204) levels have been regarded as associated with insulinoma[5], acute lymphocytic leukemia[6], human breast cancer[7] and other tumors[8]. However, more and more evidences have shown that miR-204 level was associated with an increased cardiovascular event risk. It was previously demonstrated that miR-204 inhibition attenuated aortic vascular smooth muscle phenotypic switch in diabetes mellitus[9].

Diabetes mellitus (DM) is a heterogeneous metabolic abnormality that is characterized by impaired insulin secretion, defective insulin action, or both with hyperglycemia. The chronic hyperglycemia of diabetes is associated with specific microvascular complications affecting the eyes, kidneys and nerves in the long term, in particular an increased risk for cardiovascular disease[10].
Cardiovascular disease (CVD), the leading cause of mortality worldwide, affects seriously the health of the population, which incorporate atherosclerotic CVD or special components of CVD, such as coronary heart disease, stroke, peripheral vascular disease, or heart failure. Therefore, early prediction of risk plays a critical role in evaluation and treatment of CVD.

Framingham Risk Score (FRS) are widely recognized as a preliminary screening instrument for CVD. The Framingham Heart Study (FHS) has been at the frontier of CVD epidemiology since its initiation in 1948\cite{11}. This algorithm has a gender difference, and the parameters include age, total cholesterol (TC), high density lipoprotein cholesterol (HDL), cigarette smoking status and systolic blood pressure\cite{12}. The FRS is the most convenient assessment for long-term prediction of CVD. Since diabetes mellitus is a complete risk factor of cardiovascular events, it is indispensable to predict the risk of CVD in these patients. Our research focused on the association between miR-204 and FRS, aiming to detect the moderate-and high-risk population of CVD in individuals with type 2 diabetes mellitus (T2DM).

### Method

#### Subjects

We enrolled consecutive 194 type 2 diabetic patients (average age 60.6 ± 10.0 years, 61.9% male) without known cardiovascular disease at Beijing Anzhen Hospital from January 2015 to September 2016. A fasting blood glucose level of 126 mg/dL (7.0 mmol/L) or more, glycated hemoglobin levels > 6.0%, according to WHO guidelines or by indication for insulin or anti-diabetic medications. Exclusion criteria included a history of heart failure or cardiomyopathy; no prior evidence of coronary artery disease (for example, myocardial infarction, angina pectoris, or abnormal resting electrocardiogram); kidney dysfunction, peripheral artery disease; Hepatitis B, Hepatitis C, or three times the abnormal range of liver transaminase levels; hemolytic disease; cancer; thyroid disease; and acute infection or inflammation. The study was approved by the Beijing Anzhen Hospital Ethics Committee of Capital Medical University, and all patients provided informed consent.

#### Plasma Rna Extraction

The peripheral blood specimens were collected using EDTA-anticoagulative tubes and centrifuged at 4000 RPM for 10 minutes at 4°C, and then centrifuged at 12,000 RPM for 15 minutes to completely remove the cell debris. The supernatant serum was stored at −80 °C until analysis. Use TRIZol reagent (Invitrogen, USA) to extract total RNA from the prepared plasma samples according to the manufacturer's protocols. In brief, 250µL of plasma was mixed with TRIZol reagent and chloroform\cite{13}. After centrifugation at 12,000 RPM at 4 °C for 10 min, the aqueous phase was transferred to a new centrifuge tube, and 0.8x volumes of absolute isopropanol were added. After the sample was mixed and incubated at -20 °C for 15 min, it was again centrifuged at 12000 RPM at 4 °C for 10 min. After removal of the supernatant, the precipitate was washed by adding 1.5 mL of 75% ethanol. Then the sample was
centrifuged at 12000 RPM at 4 °C for 5 min. The RNA precipitate was dissolved in 15µL of RNase-free water. The isolated RNA concentration and purity were detected by a NanoDrop 2000 system (Thermo, USA).

Quantitative Real-time Polymerase Chain Reaction

GoScript Reverse Transcription System (Promega, USA) was used to perform reverse transcription at 16 °C for 30 minutes, 42 °C for 60 minutes and 80 °C for 5 minutes to denature the enzyme. The expression levels of the miRNA-204 were quantified with a CFX Real-Time PCR Detection System (Bio-Rad, USA). Each reaction consisted of 2.5 µL cDNA, 2.0 µL primer (7.5 µmol/L), 12.5µL 2 × TaqMan Universal PCR Master Mix and 8 µL sterile water. The PCR reaction included pre-denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C if followed by 0.5 °C increments every 20 s from 75 °C to 95 °C. Since U6 was widely used as an endogenic normalizer for detecting miRNA in tissues and cells, the miRNA-204 expression was determined relative to U6 expression using the formula $2^{-\Delta \Delta CT}$. The sequences of the qRT-PCR-specific primers are as follows: mmu-miR-204-5pRT:forward 5'-GTGTATCCAGTGACGGTCTCAGTATGTTGGATACGACAGGCATA-3'; mmu-miR-204-5pF:forward 5'-GCAGTTCCCTTTGTCATCCT-3'; Hu-U6-2QF: forward 5'-CTCGCTTCGGCAGCACA-3', Hu-U6-2QR: forward 5'-AACGCTTCACGAATTTGCGT-3'. Expression of miR-204 relative to RNU-6 using the following formula: $2^{-\Delta \Delta CT}$. The measurements were performed in triplicate, and the average values were used as the detection levels. All protocols were carried out in accordance with the relevant guidelines and protocols.

Clinical And Laboratory Assessments

Each participant underwent a medical history and physical examination by a physician. Individuals who smoked regularly in the year before the examination were confirmed as cigarette smokers. Measure height and weight, and calculate body mass index (kg/m²) by dividing body weight (in kilograms) by the square of height (in meters). Blood pressure was measured on the left arm by a physician using a mercury-column sphygmomanometer, a cuff of the appropriate size, and a standardized protocol. After the patient had been sitting for at least 5 minutes, two blood pressure measurements were taken and the average was used for analysis. Biochemical parameters including serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), fasting blood glucose (FBG), and glycated hemoglobin (HbA1C) were measured by enzymatic colorimetric method.

Assessment Of The Cardiovascular Risk

Framingham Risk Score (FRS) was applied to study the risk of CVD. FRS scores were calculated based on the six coronary risk factors including age, sex, smoking, TC, HDL-C, and systolic blood pressure (SBP). The cutoff values for calculating FRS were defined as: TC < 160, 160–199, 200–239, 240–279, and ≥
280 mg/dL; for SBP: <120, 120–129, 130–139, 140–159, and ≥ 160 mmHg; and for HDL-C: <40, 40–49, 50–59, and ≥ 60 mg/dL[12]. The FRS scoring rule referred to the NCEP-ATPIII guidelines[12], calculated the corresponding scores of various parameters according to the algorithm of different genders, and finally summed up to obtain the total score, and then checked the table to obtain the corresponding ten-year heart disease risk score. The absolute percentage of CVD risk for 10 years was categorized as low risk (<10%), moderate risk (10–20%), and high risk (>20%)[12].

**Statistical analysis**

Data were analyzed with Statistical Package for Social Science (IBM SPSS version 25.0, SPSS Inc. headquarter, Chicago, USA), and expressed as frequencies and percentages for categorical variables and as the mean and standard deviation for continuous variables. Use Student's t-test and ANOVA to compare continuous variables in a normal distribution. Analyze categorical variables using chi-squared test. Spearman ρ test correlation analysis was used to examine the correlation between miRNA-204 and other cardiovascular risk factors. We used the multivariate logistic regression analysis to examine the correlation between miR-204 levels and 10-year risk for cardiovascular disease (based on FRS scores, divided into low-intermediate risk and high risk) as independent and dependent variables, respectively. For FRS different levels, the adjusted odds ratio and 95% confidence intervals were calculated. P values less than 0.05 were regarded as statistically significant. Additionally, a receiver operating characteristics (ROC) curve was plotted for circulating miRNA-204 level, SBP, and HDL-C with severe risk of FRS 10-year CVD risk, thus evaluating the ability of each variable to classify the incidence of cardiovascular disorders. The area under the curve (AUC) and 95% confidence interval of the receiver operating characteristics curve were calculated. A p value < 0.05 (two tailed) was considered significant.

**Result**

**Baseline characteristics**

Table 1 shows the baseline characteristics of our sample. Among 194 patients met inclusion criteria and were enrolled in this study (mean age 60.56 ± 10.04 years old, 58.25% male). Overall, 52.06%, 36.08%, and 11.86% of patients with type 2 diabetes were at low, intermediate, and high risk of CVD according to FRS classification. Compared with those with low or moderate risk, participants with severe CVD risk were more likely to be older and male, and have a current history of smoking. On average, participants with high risk score had higher levels of systolic blood pressure, fasting blood glucose, glucosylated hemoglobin A1c, and had lower levels of miRNA-204.
The Relationship Between Cvd Risk Factors And Frs

Table 2 summarizes the correlation between CVD risk factors and FRS 10-year CVD risk respectively by Spearman's correlation analysis. Age, gender, systolic blood pressure and miR-204 had significant correlation with FRS ($p < 0.001$), HDL-C was associated with FRS ($p = 0.020$), whereas no correlation was found with body mass index, smoking, fasting blood glucose, glucosylated hemoglobin A1c, serum total cholesterol, and low-density lipoprotein cholesterol for the study population. There was significant positive correlation between age, gender, and systolic blood pressure with FRS 10-year CVD risk.

### Table 1
Clinical characteristics of the study populations

| characteristics                  | Low risk (101) | Intermediate risk (70) | High risk (23) | P value |
|----------------------------------|----------------|------------------------|----------------|---------|
| Age, mean (SD), y                | 56.38 (9.41)   | 62.34 (7.87)           | 73.52 (4.65)   | < 0.001 |
| Male (%)                         | 37 (36.63)     | 63 (90.00)             | 13 (56.52)     | < 0.001 |
| Body mass index, mean (SD), kg/m²| 25.65 (2.78)   | 26.24 (3.01)           | 25.52 (2.13)   | 0.338   |
| Smoking, %                       | 41 (40.60)     | 41 (58.57)             | 8 (34.78)      | 0.034   |
| Systolic blood pressure, mean (SD), mmHg | 125.0 (15.0) | 131.8 (14.0)           | 141.3 (15.9)   | < 0.001 |
| Fasting blood glucose, mean (SD), mg/dL | 131.49 (35.48)  | 142.92 (47.62)       | 154.26 (54.93) | 0.039   |
| HbA1C, mean (SD), %              | 7.06 (1.03)    | 7.59 (1.70)            | 7.87 (1.70)    | 0.009   |
| Total cholesterol, mean (SD), mg/dL | 76.72 (23.60)  | 69.87 (18.90)          | 76.47 (16.04)  | 0.102   |
| High-density lipoprotein, mean (SD), mg/dL | 21.20 (11.38)  | 19.28 (5.06)           | 21.37 (3.86)   | 0.340   |
| Low-density lipoprotein, mean (SD), mg/dL | 43.23 (17.48)  | 40.46 (15.43)          | 45.38 (14.23)  | 0.371   |
| miR-204, mean (SD)               | 0.87 (0.19)    | 0.75 (0.25)            | 0.49 (0.13)    | < 0.001 |

HbA1c: glycosylated hemoglobin A1c.
respectively, however, a significant negative correlation was found in Fig. 1 between miR-204 and the FRS risk in the patients with type 2 diabetes. Figure 2 was also shown that the circulating miRNA-204 levels were negatively correlated with the FRS 10-year CVD risk ($r=-0.421 \ p<0.001$).

Table 2
Correlations of Framingham Risk Score 10-year CVD risk with demographic and biochemical parameters

| Variables          | r    | p        |
|--------------------|------|----------|
| Age                | 0.594| < 0.001  |
| Sex                | 0.406| < 0.001  |
| BMI                | 0.049| 0.499    |
| Smoking            | 0.096| 0.184    |
| sbp                | 0.432| < 0.001  |
| Fbg                | 0.066| 0.358    |
| hba1c              | 0.122| 0.090    |
| tc                 | -0.003| 0.967    |
| hdl-c              | 0.167| 0.020    |
| ldl-c              | 0.028| 0.700    |
| mir-204            | -0.421| < 0.001  |

BMI: body mass index; SBP: systolic blood pressure; FBG: fasting blood glucose; HbA1c: glucosylated hemoglobin A1c; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

Prognostic Values Of CVD Risk Factors

ROC curves were generated to obtain the prognostic values and optimal cut-off values of the miRNA as well as various CVD risk factors in Fig. 3. The miRNA-204 had an AUC of 0.884 CI(0.829–0.939) [P < 0.001]. This demonstrated that circulating miRNA-204 level can predict the high incidence risk of 10-year CVD risk by using Framingham Risk Score, and the specificity was higher than conventional risk factors SBP and HDL-C (area under the curve values were 0.746 CI(0.647–0.846) [P < 0.001] and 0.629 CI(0.530–0.727) [P < 0.05] for SBP and HDL-C, respectively).

The Association Between Risk Factors And Frs Risk Categories
Multiple logistic regression evaluated the association between risk factors for type 2 diabetes and FRS risk categories in Table 3. This results showed that age and circulating miRNA-204 levels were robust determinants of high-risk category of FRS compared with the low-intermediate risk group ($p = 0.001$).

| Variables   | Or       | 95% ci     | p value |
|-------------|----------|------------|---------|
| Age         | 2.316    | 1.063–1.385| 0.001   |
| gender      | 1.267    | 0.938–1.712| 0.123   |
| smoking     | 1.563    | 1.046–2.337| 0.029   |
| sbp         | 1.214    | 1.063–1.385| 0.004   |
| HDL-c       | 0.850    | 0.679–1.064| 0.156   |
| tc          | 1.052    | 0.979–1.129| 0.166   |
| miRNA-204   | 0.256    | 0.117–0.600| 0.001   |

SBP: systolic blood pressure; HDL-C: high-density lipoprotein cholesterol; TC: total cholesterol.

**Discussion**

In the present study, we first evaluated the association between circulating miRNA-204 levels and FRS compared with other established risk factors in clinical trials. There was a remarkable relationship between FRS risk scores and age, miR-204 levels. Moreover, based on the logistic regression analysis results, older and low circulating miRNA-204 levels make patients with type 2 diabetes more susceptible to be in high risk of cardiovascular disorders. It demonstrated that circulating miRNA-204 levels can be a novel parameter to predict the incidence of cardiovascular diseases. Therefore, high miRNA-204 levels were thought to be a protective factor of preventing CVD.

**Diabetes mellitus is associated with a high risk of cardiovascular complications**

There is no argument that individuals with DM have a significantly increased risk of macrovascular diseases. There was evidence that hyperglycemia has similar effects on endothelial cells and smooth muscle cells as hyperlipidemia[15–17]. Previous studies have demonstrated that hyperglycemia is implicated in some vascular abnormalities, such as the increase of free fatty acids and insulin resistance.
provoking decreased nitric oxide release, increased oxidative stress and subsequent inflammatory responses, disturbances of intracellular signal transduction, and activation of receptors for advanced glycation end products (AGEs) to result in macrovascular dysfunction[18–21]. Overall, T2DM is regarded as a major cause responsible for cardiovascular events.

**Microrna-204 Links To Multiple Diseases**

MicroRNAs are small, noncoding RNAs that act as a post-transcriptional regulator of gene expression and potent modulator of a variety of biological processes and pathologies[22, 23]. As one of them, miR-204 is derived from the sixth intron of the transient receptor potential melastatin 3 (TRPM3) gene. Its pathological functions have been observed in several diseases such as pulmonary arterial hypertension, diabetes, and various types of cancers.

Many studies focused on the role of miRNA-204 in cancer. In the study by Lin et al.[24], miR-204-5p overexpression induces prostate cancer cell apoptosis by repressing BCL2 expression. Zhang et al.[25] identified miR-204 act as a tumor suppressor by directly targeting ATF2 in human non-small cell lung cancer (NSCLC). Turner et al.[26] showed the opposite results by immunostaining indicating that miR-204 was previously observed an increased expression in five tumor specimens. Similarly, Zanette et al.[6] showed that miR-204 is one of five miRNAs which are most highly expressed in acute lymphocytic leukemias. Therefore, it seems to be a double-edged sword in the development and progression of tumor. Furthermore, I.C. et al.[27] reported that B-cell associated miRNA including miR-204-5p increases expression during active tuberculosis (TB).

There are also a few studies of miRNA-204 related to diabetes mellitus. Xu et al.[28] observed novel TXNIP/miR-204/MAFA/insulin pathway may be conducive to diabetes progression. And in their recent study[29], they identified serum miR-204 as an attractive novel biomarker of T1D-associated beta-cell loss in humans. In addition, Han et al.[30] suggested that miR-204-3p may play a protective role in the apoptosis and dysfunction of podocytes induced by high glucose through down-regulation of Bdkrb2. For diabetic complication, Mao et al.[31] discovered that miR-204-5p promotes the development of diabetic retinopathy by down-regulating microtubule-associated protein 1 light chain 3. Fan et al.[32] revealed the up-regulation of circKMT2E may be implicated in the pathogenesis of diabetic cataract (DC) by sponging miR-204-5p. Similarly, Gao et al.[33] described a role of miR-204 in regulating SIRT1 during the healing of diabetic corneal epithelial wounds.

**Mirna-204 May Play A Critical Role In Cardiovascular Diseases**

Cardiovascular disorders are a major characteristic of chronic inflammatory disorders such as chronic kidney disease (CKD), type 2 diabetes mellitus (T2DM), and atherosclerosis that are associated with
significant morbidity and mortality[34]. In recent years, emerging studies that miRNA-204 may play an essential role in the development and progression of cardiovascular events.

In a recent study, Yu et al.[35] showed that silencing IncRNA AK139328 significantly increased miR-204-3p expression and inhibited autophagy of cardiomyocyte, thereby reducing myocardial ischemia/reperfusion injury (MIRI) in diabetic mice. Comparably, Xiao et al.[36] discovered that ischemia-reperfusion (IR) could down-regulate miR-204, while up-regulated the proportion of LC3-II protein and autophagy cell. Torella et al.[9] described that miR-204 downregulation inhibit VSMC proliferation by targeting CAV1 in DM in vitro and in vivo. Yu et al.[37] shed light on the interaction between TUG1 and miR-204-5p in calcific aortic valve disease (CAVD), and reveal that TUG1 actively regulates post-transcriptional expression of Runx2 by sponging miR-204-5p in CAVD. Similarly, Xiao et al.[39] reported overexpression of miR-204 efficiently reversed the MALAT1-induced upregulation of Smad4 and then prevented osteogenic differentiation of human aortic valve interstitial cells (VICs). Moreover, Vikram et al.[40] elucidated that microbiome remotely regulates the expression of vascular microRNA-204 and impairs endothelial function through targeting Sirtuin1 lysine deacetylase (Sirt1). Meloche et al.[41] demonstrated in Pulmonary arterial hypertension (PAH) that coronary artery remodeling is due in part to miR-223/DNA damage/Poly[ADP-ribose] polymerase 1/miR-204 axis activation and subsequent bromodomain protein 4 (BRD4) overexpression.

In conclusion, we primarily combined serum miRNA-204 levels with the cardiovascular epidemiology in our study. It is still a challenge in patients with T2DM to timely diagnose and treat cardiovascular complications. Hence, referring to the result with FRS, circulating miRNA detection can be a novel approach to predict CVD risk.

Limitation

This study was limited due to the small sample size, which may confine the generalizability of the results. And our risk equation was derived from individuals 20 to 69 years of age and may not be generalizable to older or younger persons. Serum miRNA detection tends to be rather expensive at present leading to the limitation of our sample to some extent. Moreover, this study is a cross-sectional study with end points which are not the precise incidence of CVD and major adverse cardiovascular events. Studies are needed to validate our findings in basic research and the prospective, larger-sample random clinical trial.

Conclusion

We developed a novel risk prediction measure that estimates the long-term risk of cardiovascular events incidence in population with T2DM. Study population with lower circulating miRNA-204 levels are at a high risk for the progression of CVD. Further advances on early diagnosis, prevention and treatment for CVD are needed. Our findings suggested that reduced serum miR-204 levels are involved in CVD in
patients with T2DM. Serum miR-204 could be an independent risk factor for the CVD. MiR-204 regulation of calcification pathway may be a potential therapeutic target. However, the exact mechanisms of the association between miR-204 and cardiovascular disorders remain unclear. More study is needed to prove the relationship between miRNA-204 and CVD. The study of miRNA and its function in CVD will help us better understand its mechanism in vivo and the development of miRNA drugs targeting specific gene targets for the treatment of cardiovascular diseases.

**Abbreviations**

FRS  
Framingham risk score; ECM: extracellular matrix; VSMC: vascular smooth muscle cell calcification; T2DM: type 2 diabetes mellitus; CVD: cardiovascular disease; miR: MicroRNA; SBP: systolic blood pressure; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; HbA1c: Glycosylated hemoglobin A1c; FBG: fasting blood glucose; BMI: Body Mass Index; ROC: Receiver-operating characteristic curve; AGEs: advanced glycation end products; NSCLC: non-small cell lung cancer; TB: tuberculosis; DC: diabetic cataract; CKD: chronic kidney disease; MIRI: myocardial ischemia/reperfusion injury; IR: ischemia-reperfusion; CAVD: calcific aortic valve disease; VICs: valve interstitial cells; PAH: pulmonary arterial hypertension.

**Declarations**

**Authors’ contributions**

RW carried out the experiments, acquired the data and wrote the first draft of the paper; Y-DD carried out the experiments and wrote sections of the manuscript; Y-XZ, X-LL, HS recruited the subjects and performed the patients assessments and critically reviewed the paper for intellectual content. Y-DD, Y-QP and SZ performed statistical analyses; H-LG conceived and designed the study and handled funding and supervision. All authors read and approved the final manuscript.

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Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

All participants provided written informed consent before enrollment in this study.

Ethics approval and consent to participate

The study was approved by the Beijing Anzhen Hospital Ethics Committee of Capital Medical University, and all participants signed an informed consent.

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Figures
Figure 1

Association between Framingham Risk Score categories and miRNA-204 levels
Figure 2

Correlations of Framingham Risk Score 10-year CVD risk with miRNA-204 levels
Figure 3

Prognostic values of miRNA-204 and other CVD risk factors