

Original Article

Circulating MiRNA biomarkers serve as a fingerprint for diabetic atherosclerosis

Jing-Yun Zhang¹, Ying-Lan Gong², Chun-Jun Li¹, Qi Qi¹, Qiu-Mei Zhang¹, De-Min Yu¹

¹Key Laboratory of Hormones and Development (Ministry of Health), Tianjin Key Laboratory of Metabolic Diseases, Tianjin Metabolic Diseases, Hospital & Tianjin Institute of Endocrinology, Tianjin Medical University, 300070, Tianjin, China; ²Zhejiang University, Zhejiang, China

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Abstract: Type 2 diabetes mellitus induced atherosclerosis (DA) is regarded as a major cause of disability and death in diabetic patients. The early prediction of atherosclerosis in patients DM is necessary. Therefore, we aimed to identify special plasma microRNAs that can serve as a novel non-invasive screening signature of DA patients with atherosclerosis and test its specificity and sensitivity in the early diagnosis of DA. In total, we obtained plasma samples from 285 diabetic atherosclerosis patients and matched diabetic retinopathy (DR) patients, diabetic nephropathy (DN) patients, diabetes mellitus without complication (DM) and healthy controls. An initial screening of miRNA expression was performed through TaqMan Low Density Array (TLDA). Three miRNAs were significantly increased in patients with DA compared with other groups after the multiple stages. The areas under the receiver operating characteristic (AUC) curves of the validated three-plasma miRNAs signature in DA comparing with NC were 0.881, 0.709 and 0.842 while the merged was 0.940 while DA comparing with DM was 0.879, 0.663, 0.731 and the merged was 0.928. The three miRNA could also distinguish DA from DN with an AUC of 0.894, 0.782, 0.910 and 0.963 (merged) as well as from DR with an AUC of 0.876, 0.815, 0.850 and 0.925 (merged). In conclusion, these data provide evidence that plasma miRNAs have the potential to be sensitive, cost-effective biomarkers for the early detection of DA. These biomarkers could serve as a dynamic monitoring factor for detecting the progression of DA from DR, DN, DM patients.

Keywords: Atherosclerosis, DM, plasma miRNA, ROC, risk score

Introduction

Type 2 diabetes mellitus (DM) is associated with high increased risk for development of cardiovascular disease (CVD), including acute myocardial infarction, stroke, and peripheral artery disease [1-3]. The macrovascular pathologies in DM include a more severe development of atherosclerosis, increased arterial stiffness, and endothelial dysfunction [4, 5]. It has been identified that oxidative and glycemic stress, chronic low grade inflammation, and impaired vascular tissue repair are involved in the pathogenesis of atherosclerosis during the development of DM [6-8]. Researchers also found that plaque rupture is preceded by accumulation of pro-inflammatory and toxic lipoprotein-derived lipids, death of connective tissue-producing smooth muscle cells, and degradation of fibrous tissue [9]. DM induced athero-

sclerosis (DA) is regarded as a major cause of disability and death in diabetic patients and it has been confirmed that not all diabetic patients develop it during their lifetime [10, 11], thus, it is necessary to determine the susceptibility of AS in patients with diabetic patients and to distinguish with other distinguish of DM. Although some studies have sought to identify risk factor for the development of DA in the early stages, no clinically significant single parameter has been identified. New methods to identify DM patients at risk to develop DA from other common complication including diabetic retinopathy (DR) and diabetic nephropathy are needed.

MicroRNAs (miRNAs) identified as a class of conserved 19-25 nucleotide non-coding RNA which could regulate gene expression through a post-transcriptional approach [12-14]. Recently,

Circulating miRNA biomarker for DA

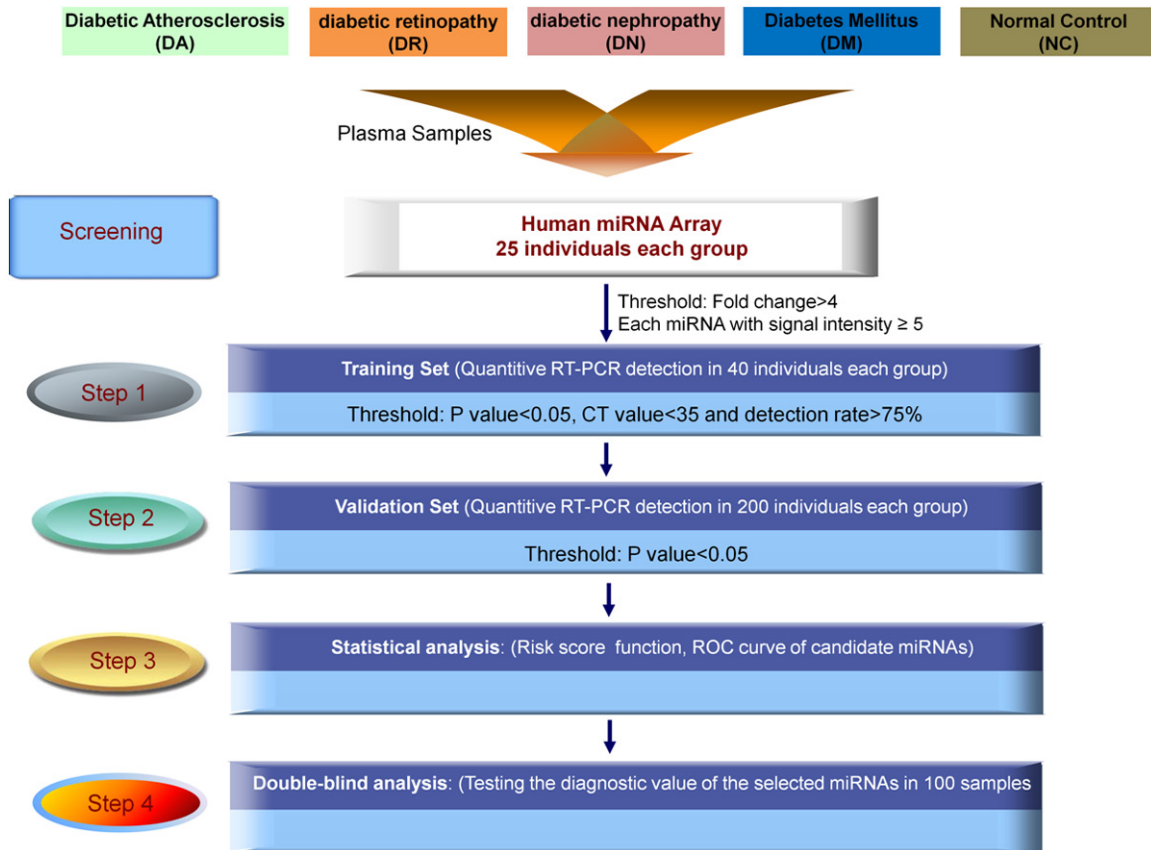


Figure 1. Overview of the workflow.

miRNAs have been demonstrated to play an important role in diabetes and its complications, regulating multiple biological pathways closely related to DA [15, 16]. Although current studies have revealed a link between the expression of miRNAs and the development of DA, these studies mainly focused on miRNAs expressed in cells or animals model. The global miRNA pattern in the sera of plasma samples of DA patients has not been determined. In addition, circulating miRNAs including plasma, serum, and other body fluids, particularly plasma miRNAs, are abundant, quiet stable. Recent studies have demonstrated that miRNAs are closely related to various diseases including cancers, congenital diseases and autoimmune disease [13, 17-19]. These findings indicated that there may be one or more special plasma miRNAs expression pattern in DA that could serve as a novel non-invasive approach for early detection of DM or other complications.

The circulating miRNA expression was screened through RT-PCR-based TaqMan low density Array (TLDA) in 25 patients diagnosed with DA

and other 25 patients with DN, DR, DM (without complication) and healthy controls were enrolled. A risk score analysis was performed to determine the potential ability of candidate miRNA in predicting DA from DN, DR, DM or healthy controls.

Materials and methods

Study design

This study was approved by the Institutional Ethics Committee of Tianjin Medical University, and a written informed consent was also obtained from each participant. The DA patients were confirmed with carotid ultrasonography described below:

Carotid artery ultrasonography was performed on each subject by the same experienced sonographer with a linear 3-10 MHz probe in B-mode (SXFL012-IU22; Philips Ultrasound, Inc.). Patients were examined in the supine position with the head tilted backwards. High-resolution images of the longitudinal lateral

Table 1. Clinical information of patients enrolled in each group

| Feature | DA | DR | DN | DM | NC | P value |
|---------------------------|-----|-----|-----|-----|-----|---------|
| All cases | 285 | 285 | 285 | 285 | 285 | |
| Age | | | | | | 0.636 |
| <60 | 85 | 92 | 77 | 91 | 90 | |
| ≥60 | 200 | 193 | 208 | 194 | 195 | |
| Gender | | | | | | 0.559 |
| Male | 177 | 181 | 175 | 169 | 188 | |
| Female | 108 | 104 | 110 | 116 | 97 | |
| Current Smoker | | | | | | <0.001 |
| Negative | 88 | 77 | 99 | 65 | 110 | |
| Positive | 197 | 208 | 186 | 220 | 175 | |
| BMI (kg/m ²) | | | | | | <0.001 |
| ≤24 cm | 101 | 112 | 97 | 119 | 155 | |
| >24 cm | 184 | 173 | 188 | 166 | 130 | |
| Diabetes Duration (years) | | | | | | 0.125 |
| ≤10 | 54 | 78 | 69 | 68 | / | |
| >10 | 231 | 207 | 216 | 217 | / | |
| Hypertension | | | | | | 0.196 |
| Negative | 207 | 216 | 194 | 211 | / | |
| Positive | 78 | 69 | 91 | 74 | / | |
| HbA1c (%) | | | | | | 0.802 |
| ≤8 | 91 | 87 | 98 | 92 | / | |
| >8 | 194 | 198 | 187 | 193 | / | |

view of the area of the bilateral common carotid arteries (CCAs), carotid bulbs (CBs), and internal carotid arteries (ICAs) were obtained according to the recommendations of the American Society of Echocardiography Carotid Intima-Media Thickness Task Force [20].

In the screening stage, 25 DA patients and 25 matched DN, DR, DM patients and healthy control plasma samples were subjected for TLDA to identify the miRNAs that were differentially expressed. Thereafter, we performed individual RT-qPCR in the training phase to further filter signals of the screened miRNAs. Subsequently, we perfected the number of plasma miRNAs included as the DA signature in a validation set including 200 samples in each group. We also analyzed another 100 samples in a blinded fashion (the investigators performing the molecular analysis on the blood samples were blinded to the patients' clinical diagnosis) to validation of the diagnostic capability of the candidate miRNAs. The protocols, including the diagnosis procedure and plasma collection methods, were identical in the two hospitals (Figure 1). The detailed information of patients and controls was listed in Table 1.

RNA isolation and RT-qPCR assay

Up to 3 ml of whole blood from each fasting participant was collected, the samples used in our study were obtained from the blood samples collected with anticoagulant tube. Total RNA was extracted by using Trizol as described before [21]. MiRNAs were reverse-transcribed with the TaqMan MicroRNA Reverse Transcription Kit (Invitrogen, CA, USA) as described by the manufacturer. MiRNA-specific TaqMan MicroRNA Assays (Invitrogen, CA, USA) was used for the plasma miRNA expression detection. The data obtained were calculated by the 2^{-ΔΔCT} method as described before. For the analysis of the expression levels of miRNAs, both the internal reference (U6) and external normalization cel-miR-39 was applied for normalization.

TaqMan low density array

MiRNA profiling was performed using TLDA (v2.0) according to the manufacturer's recommended protocol (Invitrogen, CA, USA).

Risk score analysis

Risk score analysis was performed to evaluate the associations between the concentrations of the plasma miRNA expression levels. The upper 95% reference interval of each miRNA value in controls was set as the threshold to code the expression level of the corresponding miRNA for each sample as 0 and 1 in the training set. A risk score function (RSF) to predict DA group was defined according to a linear combination of the expression level for each miRNA. For example, the RSF for sample i using information from three miRNAs was: $RSF_i = \sum 3j - 1W_j.s_{ij}$. In the above equation, s_{ij} is the risk score for miRNA j on sample i, and W_j is the weight of the risk score of miRNAj. The risk score of three miRNAs was calculated using the weight by the regression coefficient that was derived from the univariate logistic regression analysis of each miRNA. Frequency tables and ROC curves were then used to evaluate the diagnostic effects of the profiling and to find the appropriate cutoff point, and to validate the procedure and cutoffs in the next validation sample set.

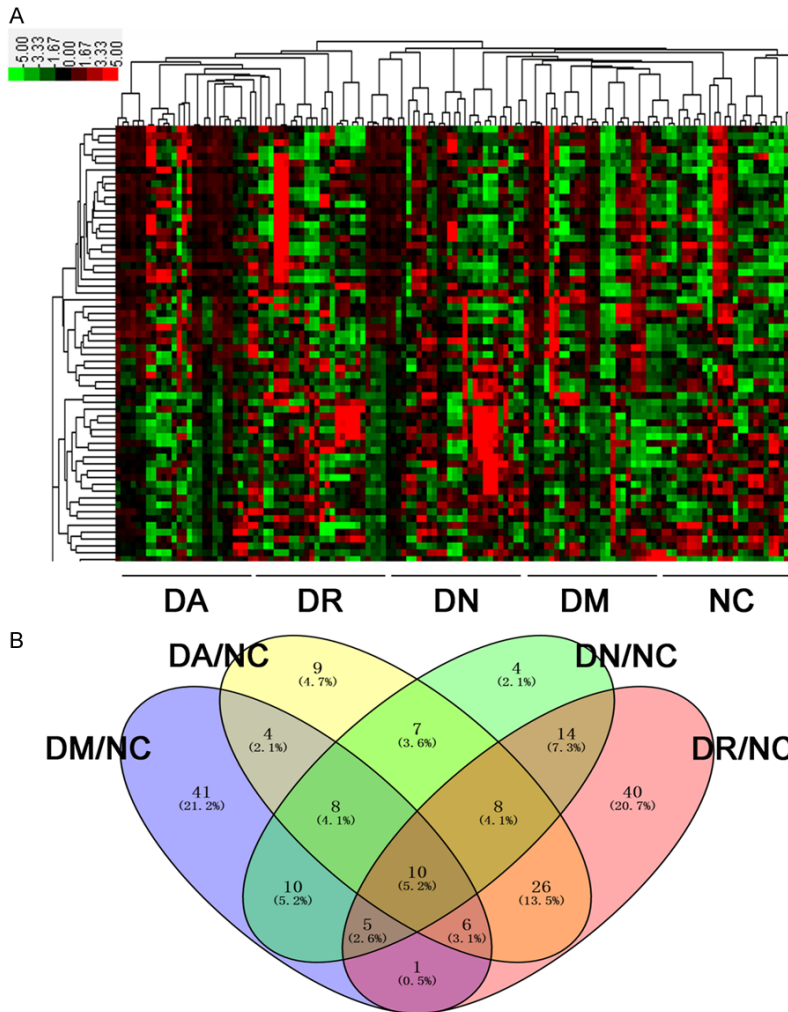


Figure 2. A TLDA-based screening of miRNAs in patient with DA and other common complication of DM. A: Cluster analysis of the different expression of the miRNAs. B: Venny analysis of different expressed miRNA in DA, DR, DN, DM and healthy control group. DA: Diabetic Atherosclerosis; DR: Diabetic Retinopathy; DN: Diabetic Nephropathy; DM: Diabetes Mellitus without complication; NC: healthy control.

Statistical analysis

The miRNA data were expressed as the mean (interquartile interval), and other variables were expressed as the mean (SD). Chi-square test analysis of variance was used to evaluate statistical differences in demographic and clinical characteristics. The nonparametric Mann-Whitney U-test was used to compare differences in plasma miRNA expression which are presented as box plot of the median and range of log-transformed relative expression level. The hierarchical cluster analysis (Average linkage) was applied by using Cluster software and Treeview. Statistical analysis was performed

using STATA 9.2, and presented with GraphPad Prism 5.0 software. Results were considered statistically significant at $P < 0.05$.

Results

Training and validation phase

In order to investigate the aberrant expression of miRNA in the plasma samples of DA patients, the TLDA technology was applied. As presented in **Figure 2A**, we found a abnormal expression of miRNA profile in DA patients comparing with the DR, DN, DM and control group. The Venny analysis was employed to further screen the special miRNA dysregulated in DA patients comparing with the healthy control while no different was obtained in DR, DN or DM comparing with the healthy control group. Finally, we obtained 9 miRNAs as presented in **Figure 2B** which indicated that the 9 miRNAs might be increased and/or decreased in DA patients and might distinguish from patients with DR, DN or DM without complication.

We further examined the 9 candidate miRNAs by RT-qPCR in a training sample set and validation set (40 samples each group and 200 samples each group). As presented in **Figure 3**, we obtained that three miRNA including miR-21, miR-218 and miR-211 was highly expressed in the plasma samples of patients with DA comparing with either DR, DN, DM without complication or healthy controls. The rest 6 miRNAs as presented in **Figure 4**, although might be different expressed in DA comparing with control group or patients was other complication, the result indicated that they might not distinguish DA from patients with all the common complication we concerned including DR, DN and DM

Circulating miRNA biomarker for DA

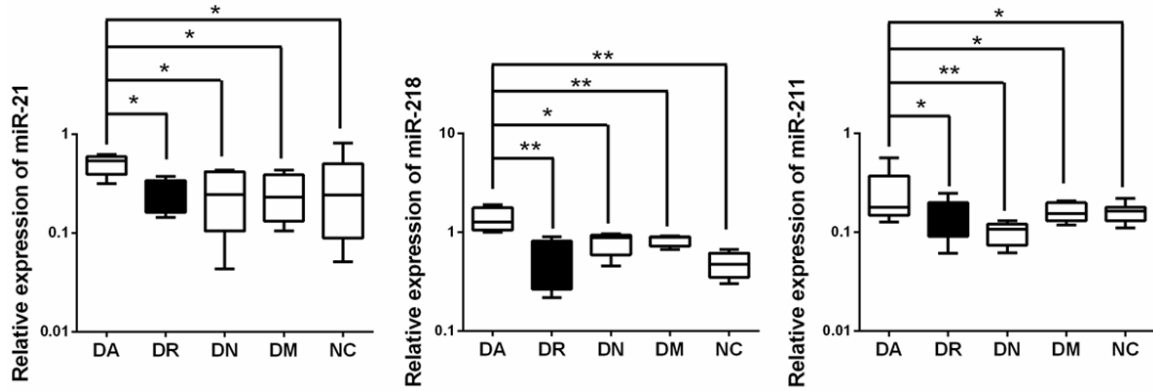


Figure 3. Different expression of miR-21, miR-218 and miR-211 in each group. Increased level of miR-21, miR-218 and miR-211 was confirmed by RT-PCR in groups. Data were presented as box plot of the mean with SD. * indicated $P<0.05$, ** indicated $P<0.01$.

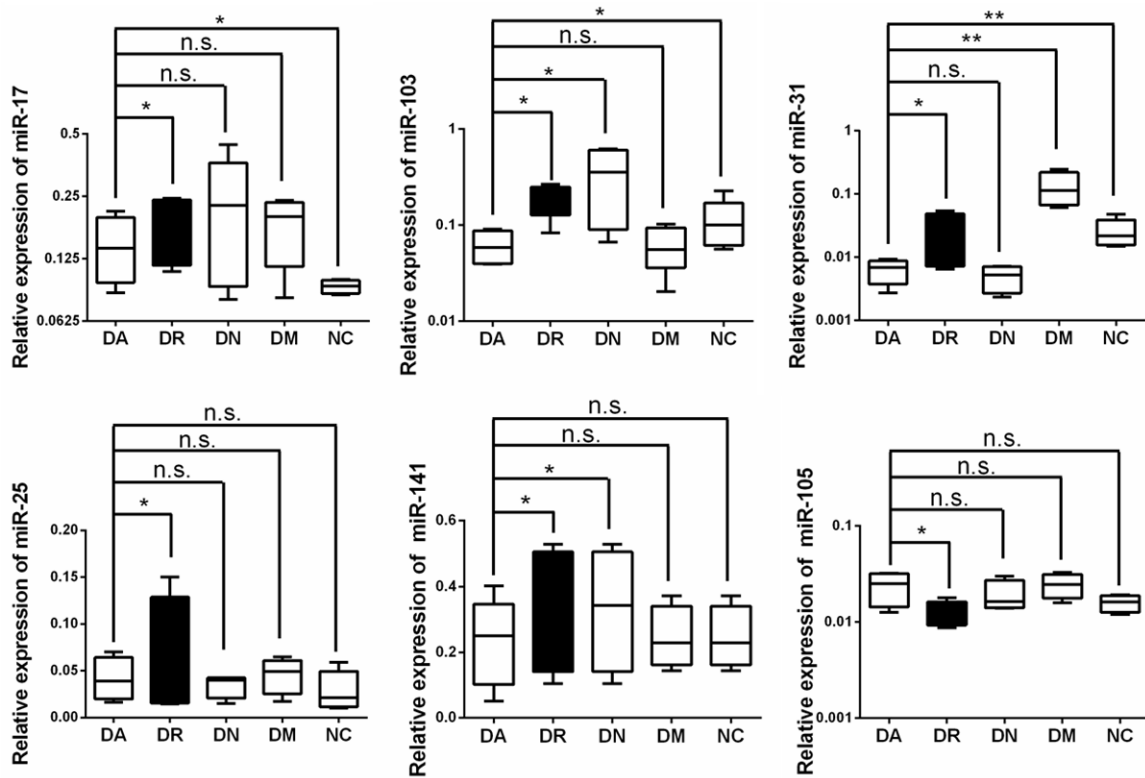


Figure 4. MiRNAs with no difference validated in each group. The expression of miRNAs were confirmed by RT-PCR in groups. Data were presented as box plot of the mean with SD. * indicated $P<0.05$, n.s. indicated no significance.

without complication, thus we could not consider the rest 6 miRNAs as potential fingerprint in DA patients.

Risk score and ROC curve analysis

To assess the diagnostic value of the three-plasma miRNA profiling system, we used a risk

score formula to calculate the risk score function for DA samples by comparing with DR, DN, DM and healthy control, respectively. ROC curves analyses were conducted to assess the diagnostic sensitivity and specificity of the three miRNAs signature for DA group comparing with the healthy control group by using risk score functions (RSFs), The areas under the

Circulating miRNA biomarker for DA

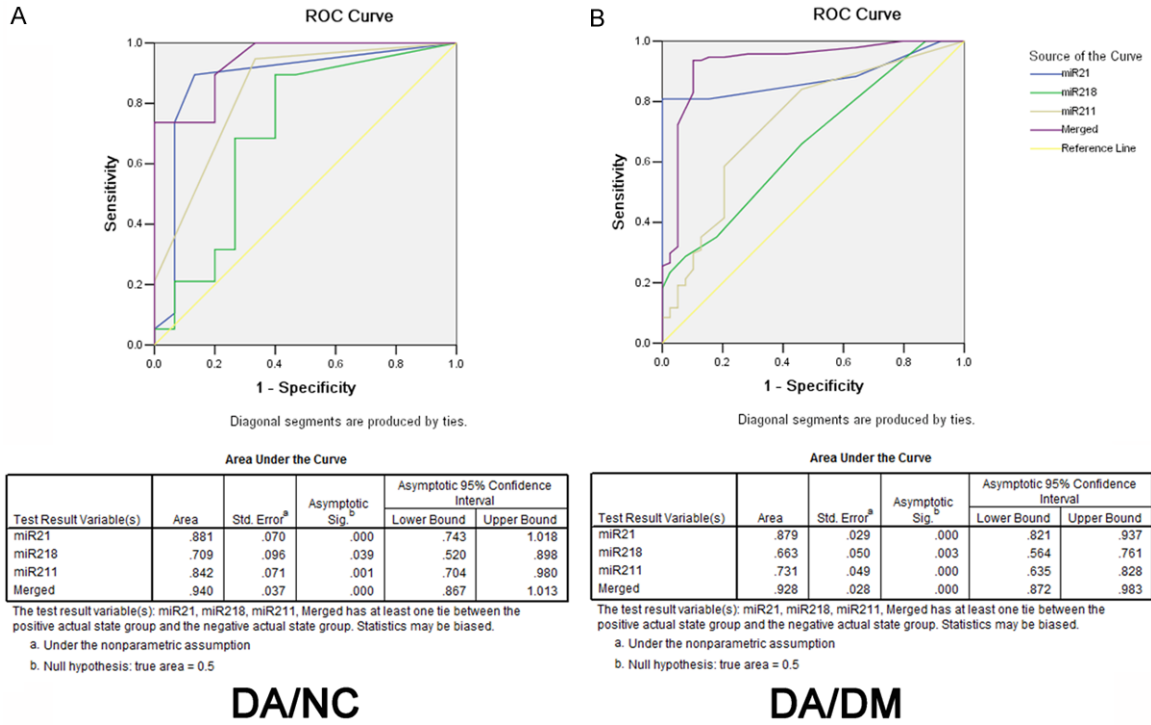


Figure 5. ROC analysis of the three potential biomarker for DA comparing with healthy control and DM group.

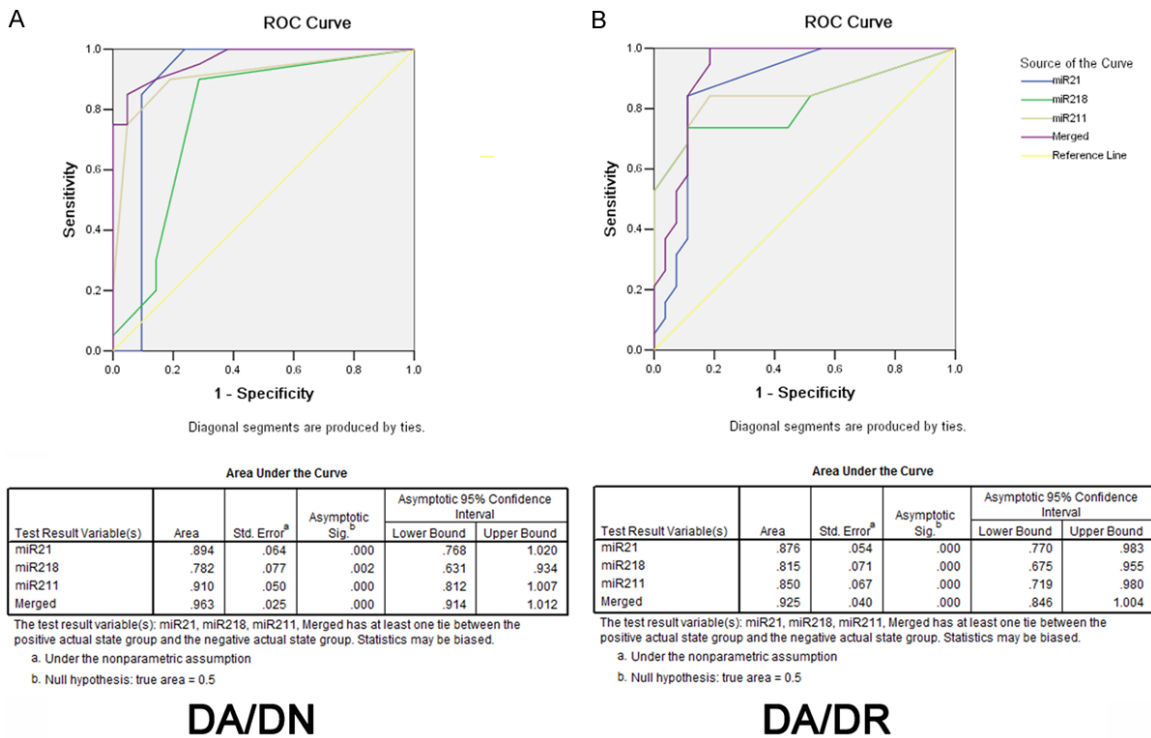


Figure 6. ROC analysis of the three potential biomarker for DA comparing with DN and DR.

curve (AUC) were 0.881, 0.709 and 0.842 for the plasma samples in validation sets. In addition,

then we compared the DA group with DM group (DM patients without complication), we

found a significant AUC of 0.879, 0.663, 0.731 and the merged was 0.928, respectively, indicating that the three miRNAs might predicting the DA from DM patients in an early stage (**Figure 5**). We further analyzed the predicting ability in DA group comparing with DM patients with other common complication including DN and DR. We found that the AUC were 0.894, 0.782, 0.910 and 0.963 (merged) and 0.876, 0.815, 0.850 and 0.925 (merged), respectively, indicating that the three miRNAs could identify the DA from DM patients with DN or DR (**Figure 6**).

Double-blind test

Another 100 plasma samples were tested in a double-blind fashion to validate the predictive ability of the three miRNA-based signatures for DA diagnosis. We used the same risk score formula to analyze the expression of the 3 miRNAs in those plasma samples and classifying them into a high-risk group and a low-risk group. On the basis of the pathologic diagnosis, the accuracy rate of the three-miRNA profile as DA signature was 84.2%.

Discussion

This study demonstrates a new fingerprint for detecting DA in DM patients for the first time. We have validated the three miRNAs including miR-21, miR-218 and miR-211 in predicting DA out of DM and DM patients with other common complications including DN and DR, which is very important for detection the progression of patients with DM developing to DA.

Diabetes is characterized by high glucose level in blood due to either less insulin secretion from pancreas or developing insulin resistance in skeletal muscle. Diabetes is categorized into many types; however, two major types of diabetes are type 1 diabetes (T1DM) and type 2 diabetes (T2DM) [22]. Patients with T2DM are in need of continuous medical care and self-management education to reduce the risk of long-term complications including atherosclerosis, diabetic nephropathy and diabetic retinopathy [23]. The process of accelerated and premature atherosclerosis in diabetic leads to an increased risk of cardiovascular events [24]. Researcher has explored long-time in detecting the early biomarker for the prediction of DA in DM patients. Among T2DM patients, an increased level of CD31+/annexin V+ MPs and

decreased CD62E+ MPs were significantly associated with asymptomatic atherosclerosis [25]. In addition, GDF-15 was also reported as a target and biomarker for diabetes and cardiovascular diseases [26]. They also identified that circulating levels of MP originated from apoptotic endothelial cell-derived were significantly increased in diabetic patients as compared with normal subjects, but level of activated endothelial cell-derived MPs was lower than in healthy volunteers indicating that they might be biomarkers for AS patients [25]. However, no systemic analysis was applied to investigate the predicting ability of these parameters in DA patients as well as DM with other complications.

It has been reported that the plasma miRNA expression profiles for various diseases may serve as fingerprints for disease detection. A plasma miRNA-based signature would make it possible to comprehensively analyze DA without or less used of other invasive procedures. This is the first investigation that demonstrates the plasma miRNA profile could be used as potential biomarker of DA. We identified three DA-associated miRNAs, including miR-21, miR-218 and miR-211. Mir-21 has been documented that negatively regulates Treg cells through a TGF- β 1/Smad-independent pathway in patients with coronary heart disease [27]. Besides, in the absence of microalbuminuria, the increased miR-21 expression in patients with iCIMT indicated this miRNA might be involved in the early stages of atherosclerotic process in hypertensive patients [28]. From a clinical view, the miRNA biomarker selected in our study for the monitoring of DM might be useful for indicating the clinical typing of DA which is a great help to decide the way of the treatment.

In conclusion, we have identified an unique miRNA biomarkers for early screening of DA, which may serve as a novel non-invasive approach for diagnosis and dynamic monitoring of DM.

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Disclosure of conflict of interest

None.

Address correspondence to: De-Min Yu, Key Laboratory of Hormones and Development (Ministry of Health), Tianjin Key Laboratory of Metabolic Diseases, Tianjin Metabolic Diseases Hospital & Tianjin Institute of Endocrinology, Tianjin Medical University, 300070, Tianjin, China. Tel: +86-22-23333206; Fax: +86-22-23333269; E-mail: 2953519320@qq.com

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