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Circulating miR-126 is a potential biomarker to predict the onset of type 2 diabetes mellitus in susceptible individuals

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is a major public health problem in China. Diagnostic markers are urgently needed to identify individuals at risk of developing T2DM and thus encouraging healthier life styles. Circulating miRNAs are valuable sources for non-invasive biomarkers of various diseases. The aim of this study was to examine whether reduced miR-126 expression could predict the onset of T2DM in susceptible individuals. Two groups of study subjects were involved, one group was diagnosed T2DM in 2013 and the other group was healthy control. To this end, our results showed that miR-126 expression were already decreased before the manifestation of T2DM. Univariable logistic regression confirmed that the plasma miR-126 level was inversely associated with the onset of DM ($P = 0.0158 < 0.05$), suggesting reduced miR-126 is a predictor for the onset of T2DM. According to the logistic regression model and ROC curve, a cut-off points of miR-126 plasma level as 35 is recommended for clinical study to predict whether an individual is more likely to develop T2DM. If miR-126 expression is lower than 35, the individual is more likely to T2DM in the next 2 years. In conclusion, our results support the notion that the circulating miR-126 can be developed into a non-invasive and rapid diagnostic tool for the prediction of susceptible individuals to developing T2DM.

1. Introduction

Despite the development of advanced medications for the prevention of diabetes mellitus (DM), unfortunately, the prevalence of Type 2 DM (T2DM) is increasing at a dramatic pace worldwide [1], especially in China. T2DM patients in China account for almost one third of DM patients worldwide [2–4] as a result of negative changes in lifestyle, such as reduced physical activity and increased nutrition. Overall, T2DM is a heavy socioeconomic burden, and becoming a major risk factor for morbidity and mortality worldwide [5].

To date, the causes of T2DM remain unclear and a definitive cure is still not available [6]. However, the onset and progression of T2DM will be drastically delayed if preventive interventions could be implemented for highly susceptible T2DM individuals before and during the initial phase of the disease. The development of biomarkers for early prediction of T2DM will help identify individuals at risk of developing T2DM, improve the care for these individuals and thus delaying the occurrence and/or reducing the severity of the disease. Hence, the discovery of new and reliable biomarkers would enable clinicians to tailor individualized preventive and therapeutic approaches and minimize the expected negative impacts of the disease.

MicroRNAs (miRNAs) belong to a category of non-coding small RNAs that consists of 18–25 nucleotide. Altered expression of miRNAs has been documented in most human diseases [7,8]. Their unique expression signatures in diseases and their stability in circulation and *ex-vivo* have prompted an increasing interest in their use as biomarkers for diagnosis and prognosis as well as potential

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therapeutic targets [9–11]. To date, miRNAs have been widely reported in blood as potential biomarkers for the detection of cancers [8,12], cardiovascular diseases [10,13], and kidney diseases [14].

Aberrant expression of miRNAs in plasma and serum samples of DM patients has been reported recently [15,16], suggesting a potential for their use as biomarkers for disease diagnosis. We previously examined the expression of a panel of plasma miRNAs in three groups of people: normal individuals (fasting glucose (FG), 4.8–5.2 mmol/L), T2DM susceptible individuals (FG, 6.1–6.9 mmol/L), and diagnosed T2DM patients (FG, ≥7.0 mmol/L) using quantitative RT-PCR (qRT-PCR). Our results Indicated that miR-126 is only miRNA that showed significantly reduced expression in susceptible individuals and T2DM patients compared to normal individuals, suggesting that miR-126 in circulation may serve as a potential biomarker for T2DM [17]. In the present study, we retrospectively recruited two groups of individuals whose glucose concentrations were normal in 2011. During the 2 years of follow-up, one group was diagnosed as T2DM, and the other group remained normal. We further investigated the predictive significance of plasma miRNA-126 levels on the onset of T2DM.

2. Materials and methods

2.1. Participants

The plasma samples of 40 Han Chinese individuals, who received routine physical exams and had normal glucose concentrations, were retrospectively retrieved at the Outpatient Department of Laboratory Medicine, Chronic Disease Hospital of NanShan District in Shenzhen, China in 2011 and 2013. They were divided into 2 study groups (20 subjects/group) based on their diagnosis in 2013: individuals with normal blood glucose levels and diagnosed T2DM patients (FG, ≥7.0 mmol/L). T2DM was diagnosed based on the combination of several parameters: FG level higher than 7.0 mmol/L, 2 h plasma glucose (PG) higher than 11.1 mmol/L in oral glucose tolerance test (OGTT). Individuals with the following conditions were excluded from the study: common diabetic complications such as retinopathy, nephropathy and cardiovascular disorders which may pose latent effect on miRNA expression. Written consents were obtained from all subjects prior to the recruitment and the study protocol was approved by the Ethics Committee of Chronic Disease Hospital of NanShan District. The clinical characteristics of the subjects are listed in Table 1.

2.2. RNA isolation and quantitative RT-PCR (qRT-PCR)

Assays to quantify the mature miRNAs were conducted as previously described [17] with minor modification. Briefly, peripheral blood was collected via venipuncture into tubes containing sodium Ethylene Diamine Tetraacetic Acid (EDTA), centrifuged at 1000 *g* for 5 min, and the plasma was carefully transferred into RNase-free tubes and stored at −80 °C until use. Total RNA containing miRNAs was isolated from 1 mL of plasma using isothiocyanate-phenol/chloroform extraction procedures. cDNA synthesis was performed with RevertAid First Strand cDNA Synthesis kit according to the manufacturer's instructions (Thermo). The SYBR® Premix Dimer-Eraser kit (TaKaRa, Shiga, Japan) was used for relative quantification of miRNAs with U6 promoter as an internal control. qRT-PCR was performed on a Roche LightCycler real-time PCR system (Roche 480 II, Switzerland).

2.3. Statistical analysis and algorithm of figure generation

Data were described as means ± SD range and P value for general characteristics of subjects. Logistic regression was performed to compare the expression of miR-126, miR-15a, miR-223, and miR-29b between DM patients and non-DM subjects. Univariate logistic regression analyses was performed to evaluate the relationships between miR-126 and the early onset of DM.

Receiver–operator characteristic (ROC) analyses were also performed with circulating miR-126 levels plotted against DM. Area under ROC curve (AUC) was estimated to assess the predictive power. In our study, AUC values indicate the ability of circulating miR-126 to distinguish DM and non-DM subjects.

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 x_1 + \ldots + \beta_k x_k = 1.1599 - 0.0331^{*} x_1$$

Based on logistic regression – a risk prediction model, the relative risk ratio for one unit decrease in miR-126 is calculated to be 0.967 for becoming diabetes versus normal. Risk model is estimated with a likelihood ratio of 0.0023, meaning the results are significant. According to the risk model and ROC curve, a cut-off point is recommended for clinical study to predict whether an individual would be more likely to develop DM in the coming years based on the expression value of miR-126. Sensitivity and specificity is also calculated based on the cut-off point selected.

SAS 9.3 software (Site 70135983. SUNY UB., USA) was used for all statistical analyses. A *P*-value of <0.05 was considered significant.

3. Results

3.1. Baseline characteristics of participating subjects

Our previous data have shown that miR-126 is significantly down-regulated in T2DM [17]. To further evaluate whether the expression of miR-126 also correlates with the onset of T2DM, we retrospectively recruited two groups of individuals with normal blood glucose levels in 2011. During the following two years, one group developed T2DM while the other group remained normal. Major characteristics of the subjects are listed in Table 1. Univariate logistic regression indicates that no statistically significant difference in age, triglyceride (TG), body mass index (BMI), systolic

Table 1
Clinical characteristics of the study subjects.

Characteristics	Normal–Normal		Normal–T2DM		*P* value
	Mean ± SD	Range	Mean ± SD	Range	
Age (year)	57.25 ± 9.64	41–72	61.20 ± 10.62	41–79	0.117
TG	1.25 ± 0.37	0.72–1.99	2.13 ± 3.05	0.30–14.83	0.077
BMI	23.90 ± 2.34	21.51–29.38	24.53 ± 2.87	18.69–30.85	0.715
Systolic pressure (mmHg)	125.50 ± 9.80	110–150	134.54 ± 16.96	110–180	0.102
Diastolic pressure (mmHg)	80.50 ± 8.91	70–96	85.77 ± 9.55	70–100	0.129
Sex					
Male	9		13		0.313
Female	11		7		

pressure and diastolic pressure was found between the two groups (Table 1).

3.2. Down-regulation of miR-126 before the onset of T2DM

The baseline levels of miR-126, as indicated by the median $2^{-\Delta CT}$, were significantly higher in individuals did not develop T2DM (N–N: Glucose is normal in both of 2011 and 2013) than in patients who developed into T2DM in 2013 (N–D: Glucose is normal in 2011, but developed T2DM in 2013) (Fig. 1). Importantly, it indicated that miR-126 expression was already altered before the manifestation of T2DM.

3.3. The correlation of circulating miR-126 with T2DM

Assessed with the univariable logistic regression model, there is a significant correlation between miR-126 and T2DM with $P = 0.0158$. Corresponding to backward selection method, T2DM is also correlated with plasma glucose concentrations, which is the most important indicator for T2DM. It implied the independent discriminatory capacity of miR-126 expression (crude odds ratio 0.967, 95% CI 0.942–0.994, $P = 0.0158 < 0.05$) (Table 2). Three other miRNAs (miR-15a, miR-223 or miR-29b) showed altered expression in T2DM patients previously [15]. However, there is no significant correlation between any of these three miRNA candidates and T2DM ($P > 0.05$) (Table 2).

3.4. miR-126 expression as a potential predictor for the onset and development of T2DM

Further, ROC curve analysis showed that miR-126 distinguishes patients with T2DM from patients with non-T2DM with an AUC of 0.8056 ($P < 0.001$) (Fig. 2).

The risk prediction model confirmed the prognostic property of miR-126. As the expression level of miR-126 decreased, patients with normal glucose level showed a significantly increased risk of becoming T2DM over time ($P = 0.013 < 0.05$). The individuals who presented with low level of miR-126 became more likely (crude odds ratio 0.534, 95% CI 0.322–0.884) to have T2DM ($P = 0.015 < 0.05$). Furthermore, if odd ratio equals to 1, the cut-off point for predictor miR-126 can be calculated as 35.0423 (relative quantification unit, $2^{-\Delta CT}$). Practically, the cut-off point can be set as 35 for clinical use. If miR-126 expression is less than 35, the patient is more likely to becoming T2DM later in life. Follow-up study for those patients whose FG are still normal in 2013, paired Student's test showed that no significant difference was found between miR-126 levels in plasma samples collected in 2011 and 2013 ($P = 0.2552 > 0.05$).

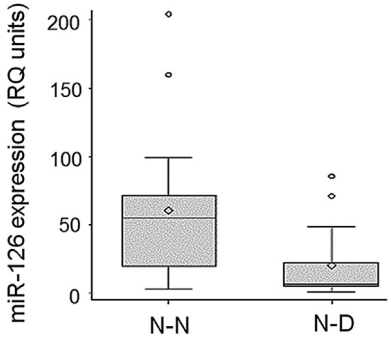

Fig. 1. Down-regulation of miR-126 expression before the onset of T2DM. Detection of miR-126 in plasma samples of individuals who maintained normal blood glucose levels in 2013 (N–N: Glucose is normal in both of 2011 and 2013) and who have developed T2DM by 2013 (N–D: Glucose is normal in 2011, but developed T2DM in 2013). The expression of plasma miR-126 was assessed by qRT-PCR. The samples with an average Ct < 40 were considered as positive. RQ, relative quantification.

Table 2
Binary logistic regression analysis for diabetes.

Variable	Estimate	SE	Odds ratio	95% Wald CI.	*P* value
Glucose	2.485	0.501	12.003	4.491–32.081	0.0001
miR-126	−0.033	0.014	0.967	0.942–0.994	0.0158
miR-223	0.005	0.011	1.005	0.983–1.027	0.6572
miR-29b	0.005	0.008	1.005	0.989–1.022	0.5146
miR-15a	0.017	0.148	1.017	0.761–1.360	0.9099

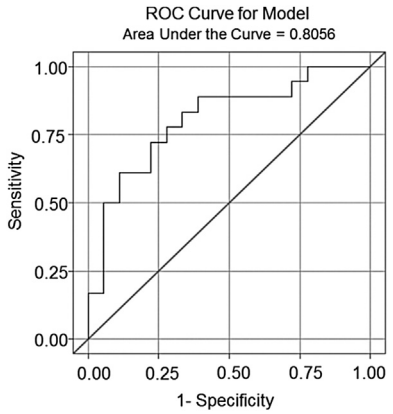

Fig. 2. ROC curve analysis for miR-126 expression levels.

Finally, the performance of a logistic regression is often evaluated in terms of its predictive ability. The sensitivity is calculated as 77.78% while specificity is 66.67%.

4. Discussion

The pandemic of T2DM is considered especially urgent in China because it is becoming a major public health problem. Despite the widely publicized report of 9.7% prevalence for diabetes and 15.5% for pre-diabetes in the 2007 survey [4], the latest epidemiologic study suggests that the estimated prevalence has increased to 11.6% for diabetes and 50.1% for pre-diabetes [2]. Unfortunately, the epidemic of diabetes and pre-diabetes in China has shown no sign of abating [2].

A long-term prospective clinical trial has shown that interventions can delay or possibly prevent the onset of T2DM [18], underscoring the importance of identifying individuals at risk to begin interventions as early as possible. Individuals at increased risk for T2DM are currently identified by a combination of traditional serum parameters (glucose, triacylglycerol, cholesterol, lipoproteins and HbA1c, etc.), physical characteristics (BMI, blood pressure, sex, etc.) and lifestyle factors (food consumption, physical inactivity, smoking, etc.). Although taking all these traditional tests and risk factors into consideration may predict the development of T2DM a few years before disease manifestation, these tests are not specific and cannot be used to assess disease susceptibility in the general population. In line with this notion, this baseline characteristic information does not substantially improve discrimination. Thus, early and lifestyle-independent predictive factors are needed to enable early intervention.

The pathogenesis of T2DM is closely linked to genetic factors. For instance, in individuals who are genetically predisposed to develop T2DM, the test is required to identify individuals at risk. The subsequent early intervention can delay hyperglycemia and the

onset of T2DM [19]. Until 2011 approximately 40 diabetes-associated genes had been identified [20], suggesting that genetic markers could potentially be used for the identification of individuals susceptible to developing T2DM later in life.

The discovery of miRNA is one of the major scientific breakthroughs in recent years and has revolutionized current cell biology and medical science. The idea of using circulating miRNAs as biomarkers is fairly new and was first proposed for the detection of various types of cancer [21]. Different diseases have been reported to present unique miRNA expression profiles. Circulating miRNAs are very stable and resistant to ribonucleases, freezing/thawing cycles and other drastic experimental conditions [22]. Consequently, serum or plasma samples can be stored at −20 °C or −80 °C for several months without notable degradation of miRNAs, which supports the utility of miRNAs as ideal candidate biomarkers [23].

Previous studies have indicated an association between miR-126 and T2DM [15,17]. Our current study further evaluated the association of plasma miR-126 levels prior to the onset of T2DM and the development of this disease. We present a novel finding that there is a cut-off point of plasma miR-126 level in individuals with normal blood glucose levels, which can predict whether they will develop T2DM in the near future or not. If circulating miR-126 expression of an individual is less than 35 (cut-off point), our prediction model suggests that he is more likely to become diabetes in the next 2 years. Importantly, our results demonstrated that miR-126 was already altered before the manifestation of T2DM, suggesting miR-126 is a predictor for the onset of T2DM. In the current study, we also noticed an association of decreased plasma miR-126 with high blood glucose. Accordingly, the miR-126 level in plasma was reduced in a glucose-dependent fashion. This association suggests that elevated plasma glucose might result in the reduced delivery of miR-126 to monocytes, which in turn contributes to VEGF resistance and endothelial dysfunction [24,25].

However, the limitations of our finding should be addressed. Firstly, the consideration of circulating miR-126 as a biomarker for T2DM is based on our results from a relatively small sample size, therefore, larger clinical studies are definitely required to further confirm our finding. Secondly, paired Student's test showed there is no significant difference in miR-126 expression between plasma samples of normal group collected in 2011 and 2013, but the levels of miR-126 in several samples collected in 2013 is lower than 35 (cut-off point). Based on our predictive model, these patients will be more likely to develop T2DM later. Due to these limitations, long time follow-up studies are needed to confirm our finding. Finally, the specificity of a single factor is poor, and the combination of multiple markers can improve predictive values [26]. Hence, the combination of existing traditional biomarkers and miR-126 should to be investigated to know whether can improve prediction for the onset of T2DM.

In conclusion, our results indicate that circulating miR-126 can be developed into a non-invasive and rapid diagnostic tool for the prediction of T2DM. Nonetheless, this present study lays the groundwork for future efforts to identify and develop miR-126 as a novel class of blood-based biomarkers for T2DM.

Conflict of interest

None.

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Transparency document

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