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Original Article

Epigenetic regulation of microRNA-375 and its role as DNA epigenetic marker of type 2 diabetes mellitus in Chinese Han population

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Abstract: Epigenetics may affect the susceptibility for type 2 diabetes mellitus (T2DM). Previously, our studies have shown that the hypomethylation of human miR-375 promoter may contribute to the pathogenesis of T2DM. However, the methylation pattern of miR-375 promoter in T2DM is not yet fully understood. In this study, the DNA methylation status of the different region of miR-375 promoter in Chinese Han population with T2DM were explored. 100 Han patients with T2DM and 100 Han healthy controls with normal glucose tolerance (NGT) were collected. Then the transcription level of pre-miR-375 and mature miR-375 were examined using quantitative real-time PCR and the methylation status of 27 CpG sites in the miR-375 promoter was determined by MassARRAY Spectrometry. The relative expression of mature miR-375 was shown as fold difference relative to miR-16 (3.0-fold, *P*=0.0260) and pre-miR-375 was markedly unregulated (2.6-fold, *P*=0.0415) in Han T2DM samples. Aberrant methylation was significantly higher within the amplicon of the miR-375 promoter in T2DMs than in NGTs, an average of 10.27% and 7.24% (P=0.0004; Figure 3A), respectively. Further, one CpG unit (CpG_26.27) was significantly hypermethylated in T2DM samples compared with NGT. Together, our results highlights for the first time that aberrant hypermethylation is a common event in Han T2DM, suggesting that the aberrant methylation of the CpG sites within miR-375 promoter may serve as a potential candidate biomarker for T2DM in the Chinese Han population.

Keywords: Type 2 diabetes mellitus, DNA methylation, microRNA-375, biomarker

Introduction

Type 2 diabetes mellitus (T2DM) is a complex disease with polygenic susceptibility, commonly resulting from defects in insulin secretion and diminished sensitivity of target tissues to insulin action [1], but the pathogenesis of T2DM has yet to be elucidated. MicroRNAs (miRNAs) are endogenous small noncoding RNAs (~22 nucleotides), which are involved in posttranscriptional control of gene expression [2]. Through their posttranscriptional regulatory mechanisms, miRNAs can directly control the expression of a large portion of the human genome and are thus involved in the regulation of major cellular activities, such as metabolism, differentiation, proliferation, and apoptosis [3, 4].

MiRNAs have already been implicated to play roles in multiple human diseases [5]. Increasing evidence have suggested that miRNAs are also involved in the pathogenesis of metabolic diseases such as diabetes mellitus. Recent studies have shown that miRNAs, including miR-375, miR-9, and miR-124a, are important regulators of specializedβ-cell functions [6-9]. It has been shown that miR-375, a pancreatic islet-specific microRNA, regulates insulin secretion through direct inhibition of insulin exocytosis [6]. High levels of miR-375 are found in the pancreatic islet of ob/ob mice and have been shown to regulate glucose homeostasis [7]. Recently, the study have shown that upregulated pancreatic miR-375 is a useful biomarker for known and novel pathways in the pathogenesis of T2DM [10]. Our previous study

have shown that the hypomethylation of human miR-375 promoter may contribute to the pathogenesis of T2DM [11]. However, the exact methylation pattern of the human miR-375 promoter in T2DM are not yet fully understood.

In recent years, researchers who are studying the role of epigenetics in the pathogenesis of T2DM [12, 13]. Epigenetic modification, especially DNA hypermethylation, are found to play an important role in the regulation of genes important for protection against T2DM. MiR-375 located in the intergenic region and has an independent promoter containing CpG islands, which provides a structural basis for regulation of its expression by methylation. In China, the prevalence rate of T2DM in the Han population is 9.26%, which is much higher than other populations in the same region [14]. This finding suggests that Han population might be a unique genetic background.

Here, in this study, we assessed the quantitative methylation status of CpG within the different region of the miR-375 promoter to determine whether the aberrant promoter methylation of miR-375 was present in Chinese Han patients with T2DM. The findings may lead to identify a new biomarker for diagnosis of T2DM in the Chinese Han population.

Materials and methods

Ethics statement

Written informed consent was obtained from all participating patients before enrollment in the study. This study was approved by the institutional ethics committee at the First Affiliated Hospital of Shihezi University School of Medicine and conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

Participants

Total 100 Han patients with T2DM and 100 Han healthy controls with normal glucose tolerance (NGT) were recruited from the Departments of Endocrinology and Metabolism at Shihezi University School of Medicine in China from 2010 to 2011. Diagnosis of T2DM was based on the World Health Organization criteria as fasting glucose ≥7 mmol/L (126 mg/dL) or the 2-hour oral glucose tolerance test glucose level ≥11.1 mmol/L (200 mg/dL) or clinical diagnosis of the disease. All the 100 Han patients with T2DM (55 male and 45 female, mean age 55.21±12.64 years) were recruited when they were hospitalized for treatment of poor glycemic control. The healthy controls (46 male and 54 female, mean age 52.16±10.26 years) were recruited from the people who had health checkup in the hospital. Any subjects possibly having any infectious diseases shortly before or during the recruitment were excluded from this study, as well as patients with autoimmune diseases.

Nucleic acid isolation

RNAs were isolated from peripheral plasma samples of Han T2DM patients and controls using the miRNeasy Mini Kit 50 (Qiagen, Germany) according to manufacturer's instruction and quantified by measuring their absorption at 260 nm. Genomic DNA was isolated from peripheral blood cells using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to manufacturer's instruction and quantified spectrophotometrically at 260 nm.

Reverse transcription and quantitative PCR of mature miR-375 and pre-miR-375

Quantitative reverse transcription-PCR (qRT-PCR) analysis of miRNAs was performed using TaqMan MicroRNA Reverse Transcription Kit and TaqMan gene-specific MicroRNA Assays (Applied Biosystems) according to the manufacturer's instructions. All measurements were performed in triplicate. MiRNA abundance is presented as threshold cycle (Ct) values normalized to miR-16 due to the stable expression in all samples in the profiling experiments. The relative abundance of miR-375 in the samples was expressed as fold change calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$).

The evaluation of reference genes for quantification of miR-375

To select a suitable reference gene, we evaluated 2 candidate targets (miR-16 and U6 snRNA) in 200 plasma samples from 100 T2DMs and 100 controls. These samples were processed under the exactly same conditions as described [15]. The sequences of all primers are given in **Table 1**.

Table 1. The qRT-PCR primers for miR-375, U6, and miR-16

Gene	Forward primer	Reverse primer
Pre-miR-375	5'-CCTCGCACAAACCGGACCT-3'	5'-GCCTCACGCGAGCCGAAC-3'
Mature miR-375	5'-AGCCGTTTGTTCGTTCGGCT-3'	5'-GTGCAGGGTCCGAGGT-3'
miR-16	5'-TAGCAGCACGTAAATATTGG-3'	5'-AGCGTCGCAGGAACATCA-3'
U6 snRNA	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

Table 2. Characteristics of study participants

Characteristic	T2DM	NGT
Gender (male/female)	55/45	46/54
Age (years)	55.21±12.64	52.16±10.26
BMI (kg/m^2)	28.82±9.35	25.35±2.90*
SBP (mmHg)	134.10±17.92	131.20±22.46
DBP (mmHg)	86.98±14.73	83.70±15.35
FPG (mmol/L)	11.40±4.42	4.12±0.45**
2 h OGTT (mmol/L)	11.34±4.17	5.16±0.42**
FINS (pmol/L)	25.27±6.73	40.72±23.06**
PINS (pmol/L)	40.25±26.12	69.56±27.28**
TC (mmol/L)	4.83±1.18	4.27±1.50*
TG (mmol/L)	2.34±1.73	1.80±1.52
LDL-C (mmol/L)	2.63±0.74	2.26±0.83*
HDL-C (mmol/L)	1.19±0.30	1.03±0.34*
HbA1C (%)	8.56±1.75	4.91±0.50**

Data are mean ± SD. NGT, Normal glucose tolerance; T2DM, Type 2 diabetes; BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; FPG, Fasting plasma glucose; 2 h OGTT, 2 h post-glucose load blood glucose; FINS, Fasting plasma insulin; PINS, 2 h plasma insulin after glucose overload; TC, Total cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol. *P<0.01, **P<0.001 compared with NGT group.

Sequenom methylation analysis

To quantify methylation levels of the different region of the miR-375 promoter to compare our previous study, the high-throughput MassARRAY platform (SEQUENOM, San Diego, USA) was carried out as described previously. [16] Briefly, bisulfite treated DNA was amplified with primers for the miR-375 CpG island. The primers were designed using EpiDesigner (http://www.epidesigner.com) as 5'-aggaagagag GGTTTTAGT TTAGTTG TAGTGT GGG-3 (forward) and 5'-cagtaatacgactcactatagggagaaggct TTTTATCTTCCAAAAATCTAACCCC-3 (reverse). The PCR products were spotted on a 384-pad SpectroCHIP (SEQUENOM, San Diego, USA), followed by spectral acquisition on a MassARRAY Analyzer. Methylation data of individual units (one to three CpG sites per unit) were generated by the EpiTyper v1.0.5 software (SEQUENOM, San Diego, USA).

Statistical methods

Significance level was set as P value <0.05. The possible differences in the miR-375 CpG methylation between Han T2DM patients and controls were tested by the Mann-Whitney and Kruskal-Wallis test. The distances between CpG methylation sites to transcription start sites have been calculated by using the RMySQL package and the SQL database version of the UCSC genome browser (http://genome.ucsc.edu/cgi bin/ hg Gateway). Two dimensional clustering has been performed by using the heatmap.2 function in the gregmisc package. Classical multidimensional scaling has been performed by using the cmdscale function and visualization was done through the scatter plot3d function in the gregmisc package. Student's t test or Fisher's exact test was used to compare the qRT-PCR results in the different groups with standard function in R statistical environment.

Results

Characteristics of the participants

Total 100 Han patients with T2DM and 100 age-matched Han healthy controls were involved in this study. The clinical characteristics of the two groups were shown in **Table 2**. The average age was 55.21±12.64 years old for patients with T2DM and 52.16±10.26 years old for healthy controls. As expected, the patients with T2DM had higher FBG, HbA1C, LDL-C, TC, TG and lower FINS, PINS, HDL-cholesterol compared with NGT controls.

The transcription level of miR-375 was upregulated in Chinese Han patients with T2DM compared with Han NGT controls

To select a suitable reference gene for quantification of plasma miR-375, we examined the levels of miR-16 and U6 by using qRT-PCR. We compared the stability of miR-16 and U6 in plasma, and found that miR-16 levels did not

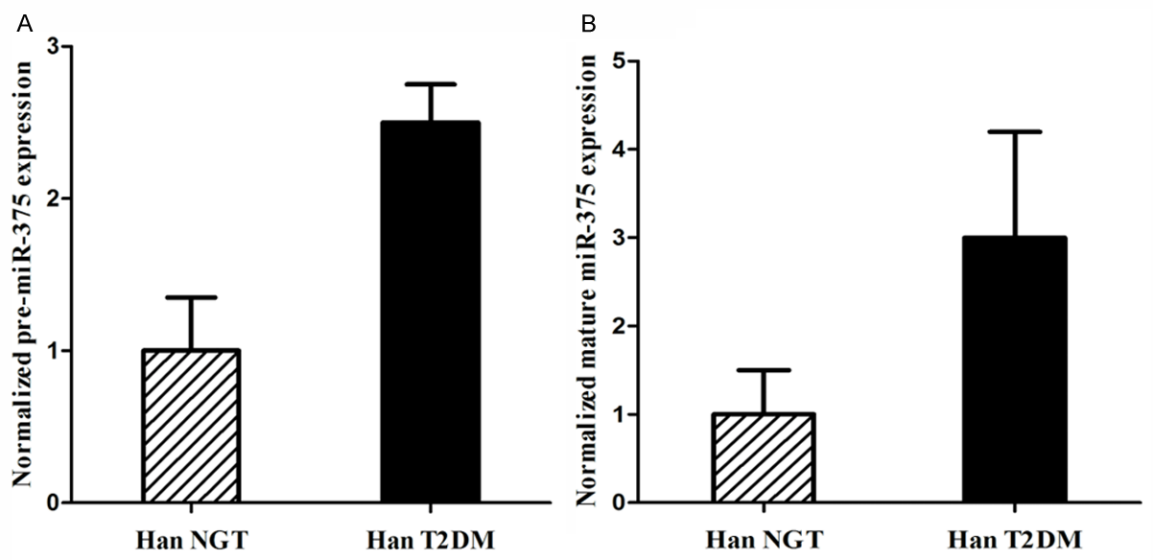

Figure 1. The transcription level of mature and pre-miR-375 in Han NGT samples and T2DM samples. The expression of pre-miR-375 (A) and mature miR-375 (B) were examined by qRT-PCR. Pre- and mature miR-375 was normalized to miR-16. Data were shown as mean ± standard deviation (SD). **P*<0.05.

change after prolonged incubation time at room temperature. However, U6 appeared to be less stable than miR-16. Therefore, miR-16 was selected as the normalization control as it displayed higher stability and abundance than U6. Here we tried to determine if aberrant transcription level of mature miR-375 was present in the Chinese Han patients with T2DM. Quantitative real-time PCRs were performed to investigate the transcription level of miR-375 mRNA in Han T2DM plasma samples and corresponding NGT samples. The relative expression of mature miR-375 was shown as fold difference relative to miR-16 (3.0-fold, *P*=0.0260, as shown in **Figure 1A**) and found that pre-miR-375 was markedly unregulated (2.6-fold, *P*=0.0415, as shown in **Figure 1B**) in Han T2DM samples.

The specific CpG hypermethylations of miR-375 promoter may contribute to its overexpression in Han patients with T2DM

We carried to detect methylation Patterns of miR-375 CpG island and performed quantitative high throughput analysis of DNA methylation by the MassARRAY system within the miR-375 promoter from -707 bp to -1,056 bp relative to the transcription start site. The methylation status of miR-375 promoter was studied in all the samples collected from T2DMs (n=86) and normal controls (n=100). A 267 bp region of the miR-375 promoter containing 27 CpG sites which could be divided into 9 CpG units were examined by MassARRAY system. Among these units, six CpG units (22 CpG sites) did not yield successful measurements. Three CpG units resulted in quantitative measurements of methylation for 90% of the samples analyzed in a high-throughput. The average DNA methylation frequency ranged from 2.5% to 24.0% in T2DMs and from 2.5% to 14.0% in normal controls. Aberrant methylation was significantly higher within the amplicon of the miR-375 promoter in the T2DMs than in normal controls, an average of 10.27% and 7.24% (*P*=0.0004; **Figure 3A**), respectively. We also generated a receiver operator characteristic (ROC) curve to assess the clinical utility of miR-375 methylation for the prediction of T2DM. It was highly discriminative between the T2DM patients and NGT samples (area under the curve =0.7438; 95% CI, 0.6226 to 0.8650; *P*=0.0004771, **Figure 3B**).

We explored if methylation of specific critical CpG units could potentially account for increased miR-375 expression in the Han T2DM samples. Analyses of the individual CpG units in the T2DM cases and controls revealed that hypermethylation were seen in one CpG units in this amplicon by MassARRAY EpiTyper. Moreover, significant differences were detected in two specific CpG sites, including CpG_26.27,

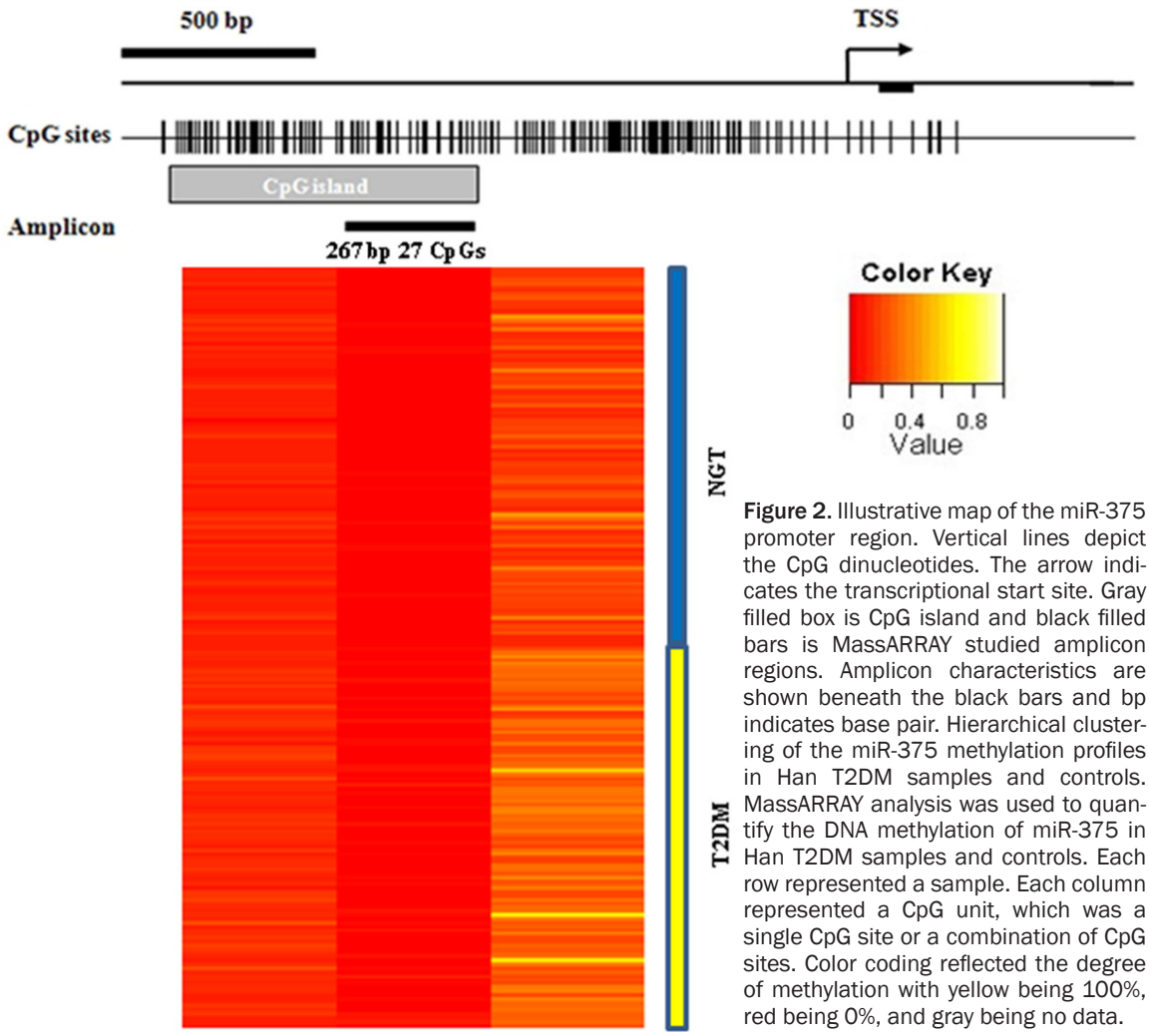

Figure 2. Illustrative map of the miR-375 promoter region. Vertical lines depict the CpG dinucleotides. The arrow indicates the transcriptional start site. Gray filled box is CpG island and black filled bars is MassARRAY studied amplicon regions. Amplicon characteristics are shown beneath the black bars and bp indicates base pair. Hierarchical clustering of the miR-375 methylation profiles in Han T2DM samples and controls. MassARRAY analysis was used to quantify the DNA methylation of miR-375 in Han T2DM samples and controls. Each row represented a sample. Each column represented a CpG unit, which was a single CpG site or a combination of CpG sites. Color coding reflected the degree of methylation with yellow being 100%, red being 0%, and gray being no data.

which were hypermethylated in the Han T2DM samples compared with the NGT samples (**Figure 3C**).

In our study, we used an unsupervised two-way hierarchical clustering of the CpG unit methylation and the T2DMs and normal controls (**Figure 2**). The patterns we observed in the cluster analyses show that methylation patterns of normal controls are notably different from those observed in T2DMs.

Correlation between the methylation level of CpG units and clinical features

In addition to finding evidence of the relationship of the methylation level of CpG units and clinical features, we performed a correlation analysis of the methylation level of CpG units and clinical features using a linear regression. Analysis of three CpG units demonstrated that the methylation level of CpG_26.27 was negatively correlated with HOMA-IS, and FINS, but positively correlated with HOMA-IR, LDL, TG and SBP (see in **Figure 4**).

Discussion

Emerging evidences have shown that the expression levels of miR-375 play a multifaceted role in various cancers [17, 18]. In this study, we sought to determine it's the expression pattern of miR-375 in the Chinese Han patients with T2DM. Here, we found that the transcription level of mature miR-375 was significantly upregulated in the Han T2DM samples compared with matched NGT samples. It had been shown previously that overexpression of miR-375 downregulated the expression of myotrophin, a known regulator of catecholamine release [19]. As hairpins are generated in the

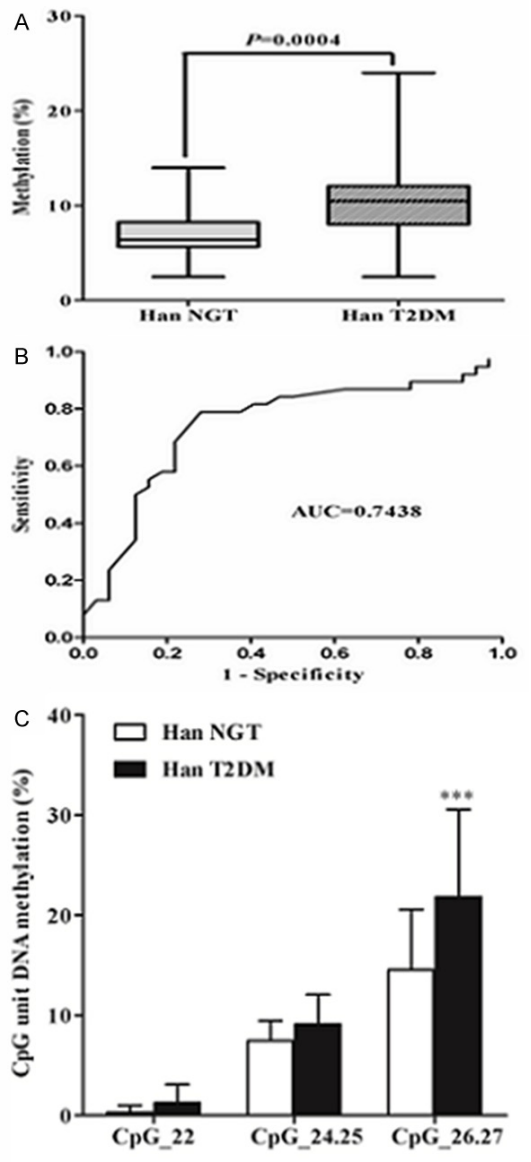

Figure 3. Methylation of the miR-375 in samples from Han patients with T2DM and NGT controls. A. The DNA methylation level of the miR-375 region was measured by MALDI-TOF MS analysis of DNA extracted from samples from 100 Han patients with T2DM and 100 NGT controls. The mean methylation levels of the 5 CpG residues in the 100 samples were used to represent the promoter methylation levels. Data were shown as mean ± standard deviation (SD). B. ROC curvefor the miR-375 methylation level in T2DM patients and NGT controls. C. Comparison of specific miR-375 CpG methylation in Han T2DM samples with NGT sample. The average methylation of specific CpG units of amplicon in 100 T2DMs and 100 NGT controls were determined by MassARRAY EpiTyper. ***P<0.001 and *P<0.05 when T2DMs compared with NGT controls by Mann-Whitney and Kruskal-Wallis test. The error bars represented standard error.

nucleus, pre-miRNAs are causally upstream of mature miRNAs, and hence are faster to react. El Ouaamari et al [20] demonstrated that pre-miR-375 was depleted by elevated glucose levels in INS-1 cells and islets. In our study, by using qRT-PCR, we also observed that pre-miR-375 expression was significantly increased in T2DMs. Together, these observations might suggest that miR-375 involved in the pathogenesis of T2DM. Since the plasma level of miRNAs can be present in a remarkably stable form and the expression level of plasma miRNAs is reproducible and consistent among individuals, earlier study demonstrated the presence of circulating miRNAs and their potential use as novel biomarkers of diseases [21]. Our data suggested that the plasma level of miR-375 might be used as a novel biomarker of T2DM in the Chinese Han population.

Epigenetic modification of DNA, such as methylation, is thought to play a key role in T2DM progression [12, 13] and miRNAs were epigenetically regulated by DNA methylation [22]. To date several studies had revealed that the expression of miR-375 was epigenetic regulated in gastric and breast cancers [17, 18], but no studies have directly linked the miR-375 promoter methylation to T2DM. In current study, we further tried to determine whether the up-regulation of miR-375 was also mediated by epigenetic mechanisms in the Chinese Han patients with T2DM. Using MALDI-TOF MS, we evaluated methylation patterns at multiple CpG sites within the promoter regions of miR-375 and found that overall higher methylation in the Han T2DM samples than NGT samples. Considering the great variations of the methylation levels of different CpG units, we further evaluated the methylation status of specific critical CpG units. Our results showed statistically significant hypermethylation in one specific CpG units from the Chinese Han T2DM samples compared with NGT samples. In the current study, the methylation of CpG_26.27 was negatively correlated with HOMA-IS, and FINS, but positively correlated with HOMA-IR, LDL, TG and SBP. Thus, these observations suggest the hypermethylation of miR-375 could be added to the known risk factors to predict the progression of T2DM in the Han population. Meanwhile, clinical detection of upregulated miR-375 levels may not be limited to using

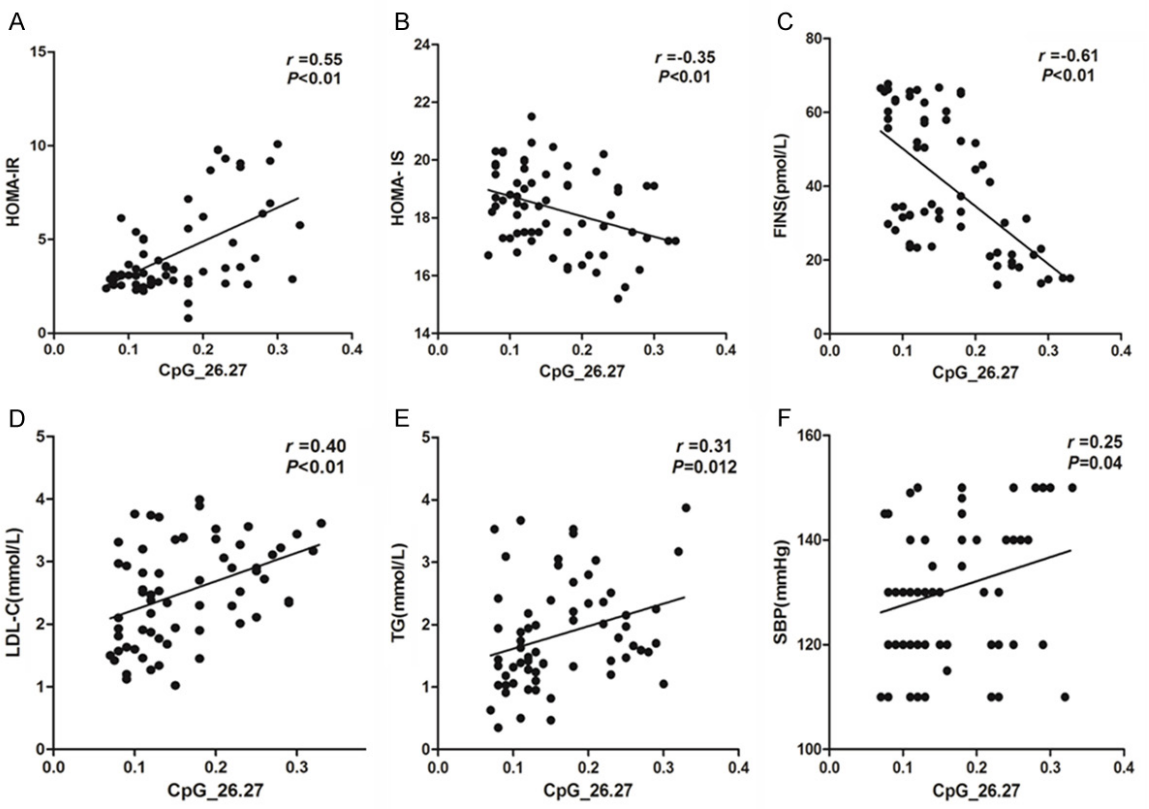

Figure 4. Correlations between the DNA methylation of CpG units and clinical features. SBP, systolic blood pressure; FINS, fasting plasma insulin; TG, triglyceride; LDL-C, Low-density lipoprotein cholesterol.

plasma for T2DM diagnosis. It would be interesting to investigate whether miR-375 is present in other body fluids, such as saliva or urine, in order to establish a possible general diagnostic method. It was known that miR-375 can inhibit insulin secretion and decrease the number of β-cells. In addition, miR-375 can affect cell proliferation, differentiation and the expression of apoptosis related genes so as to promote the occurrence of apoptosis. Therefore, we hypothesized that the hypermethylation of miR-375 gene promoter might be involved in the development of T2DM in the Han population.

Our previous study demonstrated that the CpG methylation status of miR-375 is hypomethylated, in the Han patients with impaired glucose tolerance, which may regulate the expression of miR-375 [23]. But in this study, our results showed that the miR-375 promoter is hypermethylated and did not show that the methylation status of miR-375 was negatively correlated with the transcription level of miR-375. The inconsistence of the results may be due to the different region of the promoter of miR-375 and limited sample size in this study. However, the current investigation has widened the analysis region of the promoter for studying miR-375 methylation using MALDI-TOF MS technology and warrant further studies using larger sample sizes of T2DM in the Han population.

In conclusion, our study suggests for the first time that aberrant hypermethylation is an important event in the Han T2DM population. The aberrant methylation of the CpG sites within miR-375 promoter may serve as a potential candidate biomarker for the Han T2DM population.

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Disclosure of conflict of interest

None.

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