Original Research *Highlight article*

hsa-miR-607, lncRNA TUG1 and hsa_circ_0071106 can be combined as biomarkers in type 2 diabetes mellitus

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Impact statement

T2DM is a chronic disorder that leads to alterations in gene regulation. Expression of some ncRNAs have significant changes in diabetics compared with general population. Importantly, ncRNAs have conservative and stable characteristics so that they can be used as biomarkers. This article used scientific methods to find some potential markers and tried to diagnose T2DM jointly. It can provide some evidence for ncRNAs to become a diagnostic method for T2DM in the future.

Abstract

Type 2 diabetes mellitus (T2DM) is a multifactorial disorder that leads to alterations in gene regulation. ncRNAs have the characteristics of tissue specificity, disease specificity, timing specificity, high stability and post transcriptional regulation effect. These preconditions are more conducive to promote ncRNA to become a new biomarker for clinical diagnosis. Our study aims to explore the relationship between circRNA, lncRNA, miRNA and T2DM, and to evaluate their diagnostic value for T2DM. A total of 101 pairs of T2DM and controls were conducted in the study. QRT-PCR was used to study the differential expression of circRNAs, miRNAs and lncRNAs. ROC curve was used to estimate their diagnostic value in T2DM. Compared with healthy controls, the expression levels of hsa_circ_0071106, hsa_circ_0000284, hsa_circ_0071271, hsa-miR-29a-5p, hsa-miR-3690, hsa-miR-607, lncRNA MEG3 and lncRNA TUG1were higher in T2DM (all $P < 0.05$). The AUCs of hsa_circ_0071106, hsa-miR-607 and lncRNA TUG1 for diagnosis of T2DM were 0.563,0.645 and 0.642, respectively. The combined AUC of hsa-miR-607, lncRNA TUG1 and hsa_circ_0071106 was 0.798 ([0.720~0.875], $P < 0.001$). Moreover, the sensitivity of combined diagnosis was 75.2% and the specificity was 100.0%. The levels of lncRNA TUG1, hsa-miR-607 and hsa_circ_0071106 in peripheral blood have potential clinical diagnostic value for T2DM.

Keywords: T2DM, miRNA, lncRNA, circRNA, ROC

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Introduction

Type 2 diabetes mellitus (T2DM) is a complex, multifactorial disease which may leads to a series of serious complications, such as retinopathy, diabetic nephropathy and diabetic foot.[1] At present, the trend of diabetes is increasing.[2] So it is urgent to diagnose, prevent and intervene diabetes.

Genome-wide identification of genetic risk sites shows that epigenetic effects may play an important role in the development of T2DM, and most single nucleotide polymorphisms (SNPs) are located in non-coding regions of the human genome.[3] Non-coding RNAs (ncRNAs) including long non-coding RNA (lncRNA), circular RNA (circRNA), and microRNA (miRNA) have been reported to participate in pathological developments of T2DM through various mechanisms. For example, Zhang and colleagues have demonstrated loss of miR-182 leads to impaired glucose metabolism and miR-182 regulates glucose utilization by modulating PDHC activity via FoxO1/PDK4.[4] Yang el.at. found 441 lncRNAs (366 upregulated and 75 downregulated), 683 circRNAs (354 upregulated and 329 downregulated), 93 miRNAs (63 upregulated and 30 downregulated), and 2935 mRNAs (1156 upregulated and 1779 downregulated) were remarkably differentially expressed in the T2DM group.[5] Lin el.at. constructed a T2DM-related competing endogenous RNAs (ceRNAs) network to explore the biological function of lncRNA during the development of diabetes mellitus.[6] These results mean ncRNAs can be used as a candidate diagnostic marker for T2DM.

Among the ncRNA, circRNA is a class of evolutionary conserved, stable, endogenous one. Its main feature is the presence of a covalent bond that can connect the 3'and

5′ends by back splicing. Many studies have found that some circRNAs can be used as diagnostics biomarkers of pre-diabetes and T2DM, such as hsa_circ_0054633, hsa_circ_0071106.[7,8] miRNAs are called microRNAs, which are small single-stranded RNA molecules with only 14–25 nucleotides in length. The genetic regulation involved in miRNA has been found in more and more studies. Over expression of miR-375 suppressed glucose-induced insulin secretion, and conversely, inhibition of endogenous miR-375 function enhanced insulin secretion.[9] MiR-1299, miR-126-3p, miR-30e-3p, and miR-182-5p were significantly highly expressed in individuals with prediabetes.[10,11] lncRNA, another kind of ncRNA with lengths of over 200 nucleotides, has widely implicated in the regulation of gene transcription. And Zhu *et al.*[12] have demonstrated that lncRNA MEG3 promoted hepatic insulin resistance by serving as a ceRNAs of miR-214 to facilitate ATF4 expression. All the results intimate that circRNA, lncRNA and miRNA be used as a candidate diagnostic marker for T2DM.

Currently, ncRNAs are receiving increasing attention in diabetes research. Although numerous studies have constructed diabetes-related circRNA-miRNA-lncRNA networks, most of these studies did not evaluate ncRNA together as potential biomarkers. This study is to understand the feasibility of circRNA, miRNA and lncRNA as a new biomarker for clinical diagnosis.

Materials and methods

Design and sample collection

A total of 105 T2DM patients and 105 control group subjects were recruited from the healthy screening population over 18 years of age in Huadu District, Guangzhou in 2019. According to the "China T2DM Prevention and Treatment Guidelines (2017 Version)," the diagnostic criteria of a T2DM patient were as follows: (1) fasting plasma glucose (FPG) $\geq$ 7.0 mmol/L, (2) 2 h postprandial blood glucose $\geq$ 11.1 mmol/L, (3) glycated hemoglobin HbA1c $\geq$ 6.5%, or (4) a previous history of diabetes or have typical symptoms of diabetes such as multiple drinks, polyphagia, polyuria and unexplained weight loss, with random blood glucose values $\geq$ 11.1 mmol/L. All samples excluded patients with other types of diabetes such as type 1 diabetes, gestational diabetes, and patients with cerebral infarction, myocardial infarction, malignant tumors, and chronic inflammation. Among them, 4 T2DM patients and 4 control individuals were subjected to circRNA chip analysis and other subjects were used for quantitative real-time PCR validation.

Data were recorded with epidemiological questionnaire surveys (demographic characteristics, smoking, drinking behavior and past disease history, etc.), and physical measurements (blood pressure, etc.) and laboratory tests (fasting blood glucose (FBG), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and other biochemical indicators) for analysis. All subjects collected 3 ml of whole blood using a vacuum blood collection tube containing EDTA anticoagulant in the morning on an empty stomach. Then they were centrifuged at 1500 rpm and stored at −80°C for further processing.

RNA extraction

Stored serum samples from study participants were thawed. RNAiso Blood extraction reagent (Takara Bio Inc, Japan) was used to extract total RNA from 400 ul of whole blood according to manufacturer's instructions. The concentration and purity of total RNA were determined by measuring the A260/208 ratio (1.8~2.1) using a Nano Drop-2000 (Thermo, USA).

Candidate circRNA and target miRNA and lncRNA prediction

Based on circRNAs' own characteristics and previous research experience, our research began to explore from circRNA. Arraystar Human circRNA Arrays v2 provided by Arraystar was used to perform the peripheral blood of 4 diabetes and 4 control groups for microarray analysis. The circRNA chip experiment was assisted by Shanghai Kangcheng Biological Co., Ltd. The microarray results showed that a total of 798 circRNAs were screened, of which 296 were up-regulated and 502 were down-regulated. The candidate circRNAs were selected from the 798 circRNAs according to the following criteria: fold changes $\geq$ 2.0, exon and the original intensity $>$ 200, and P value $<$ 0.01. hsa_circ_0071106, hsa_circ_0071271, hsa_circ_0000284, and hsa_circ_0003344 were selected for validation by the qRT-PCR. To ensure that they were significantly different between the T2DM group and the control group, we also used the ΔC_T($\Delta C_T = C_{T,\ Target} - C_{T,\ internal\ reference}$) method to calculate relative expression.[7] Then circBank (http://www.circbank.cn/), starBase (http://starbase.sysu.edu.cn/) and circIteractome (https://circinteractome.nia.nih.gov/) databases were used to predict the miRNA interacting with hsa_circ_0071106. And the relationship between miRNAs were described by a network diagram using Cytoscape software and the results are shown in Figure 1. In addition to preferentially selecting those predicted by the two databases, we also selected the miR-29 family that has been repeatedly shown to be associated with T2DM.[13,14] After consulting literatures, hsa-miR-607, hsa-miR-29a-5p, hsa-miR-3690, and hsa-miR-409-3p were determined to conduct further research.[14–17] Subsequently, the lncRNA interacting with the related miRNAs which were selected above was predicted by starBase and DianalncRNABase(http://carolina.imis.athena-innovation.gr/diana_tools/web/) bioinformatics. Using Venn diagrams to get their intersection.

Quantitative real-time PCR validation

Four circRNAs, four miRNAs and three lncRNAs were selected for population qRT-PCR experiments. Prime ScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time)(Takara Bio Inc, Japan) was used to remove DNA. Reverse transcription reaction system: 5×gDNA Eraser Buffer 2.0 ul, gDNA Eraser 1.0 ul, add the appropriate amount of total RNA and makeup to 10 ul with enzyme-free water, react at 42 °C for 2 minutes. circRNA, miRNA, and lncRNA used, respectively, TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) Fluorescence Quantitative Detection Kit (Takara Bio Inc, Japan), Mir-x miRNA First-Strand synthesis kit (Clontech, China), and

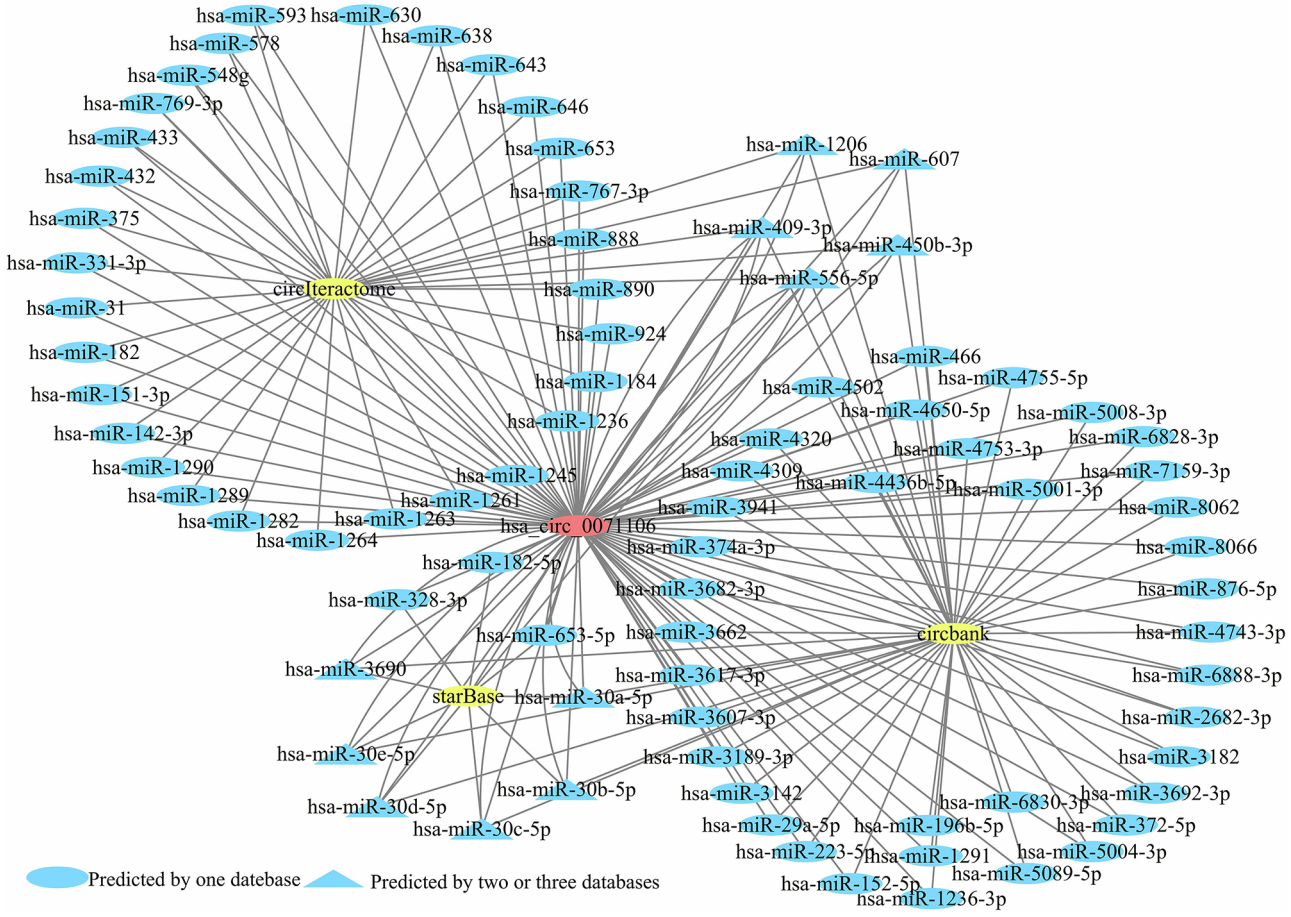

Figure 1. hsa_circ_0071106-miRNA interaction network. Red represents hsa_circ_0071106, blue represents predicted miRNA, yellow represents database. (A color version of this figure is available in the online journal.)

PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio Inc, Japan) to reverse transcription of the extracted RNA to cDNA according to the manufacturer's instructions. The qRT-PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The qRT-PCR was performed in a 20 μl reaction volume, including 10 μl TB Green Premix Ex Taq (Tli RNaseHPlius), 0.8 μl PCR Forward Primer (10 μM), 0.8 μl PCR reverse primer (10 μM), 0.4 μl ROX, 2 μl cDNA and 6 μl nuclease-free water. Reaction conditions: initial denaturation at 95°C for 34 s; followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 34 s and finally the dissolution curve analysis was at 95°C,15 s, 60°C, 15 s, 95°C, 15 s. Each sample is in triplicate. U6/GAPDH were used as the internal reference. The $2^{-\Delta\Delta Ct}$ method was used to detect relative expression of RNA, Eq. is as follows.[18] Table 1 is the primer sequence of target RNA, U6 and GAPDH.

$$\Delta C_T = C_{T,\text{Target}} - C_{T,\text{internal reference}}$$

$$\Delta\Delta C_T = \Delta C_{T,T2DM} - \Delta C_{T,\text{Control}}$$

$$\text{amount of target} = 2^{-\Delta\Delta C_T}$$

Table 1. Quantitative real-time PCR primer sequence.

Primer	Primer sequence (5'to3')
hsa_circ_0071106 F	GAAGCTGCTGATCGGAAGAAA
hsa_circ_0071106-R	GCCGGTTCTGCTCTACTTGG
hsa_circ_0071271 F	GGAACCCAAAGACCTGCTACAA
hsa_circ_0071271-R	TGGTCCACTCCAGCTCCTCT
hsa_circ_0003344 F	GGAAGAGCTACGGGAGATCAA
hsa_circ_0003344-R	CCAGGTCTCCCTTATCGACCT
hsa_circ_0000284 F	ATAGACTTTGGGTCGGCCAGT
hsa_circ_0000284-R	TCTTCACACTACAAAAGGCACTTGA
hsa-miR-3690	ACCTGGACCCAGCGTAGACAAA
hsa-miR-607	CGCGCGGTTCAAATCCAGATCTATAAC
hsa-miR-409-3p	GAATGTTGCGTGAACCCCT
hsa-miR-29a-5p	ACTGATTTCTTTTGGTGTTCAG
lncRNA MEG3 F	CTGCCCATCTACACCTCACG
lncRNA MEG3-R	CTCTCCGCCGTCTGCGCTAGGGGCT
lncRNA TUG1 F	TAGCAGTTCCCCAATCCTTG
lncRNA TUG1-R	CACAAATTCCCATCATTCCC
lncRNA MALAT1 F	AACGCAGACGAAAATGGAAAGA
lncRNA MALAT1-R	CCTTCTAACTTCTGCACCACCAGA
U6 F	CTCGCTTCGGCAGCACATATACT
U6-R	ACGCTTCACGAATTTGCGTGTC
GAPDH-F	GCACCGTCAAGGCTGAGAAC
GAPDH-R	TGGTGAAGACGCCAGTGGA

Table 2. Demographic and biochemical indexes characteristics of study subjects.ww

Variable		T2DM ($n=101$)	Control ($n=101$)	$t/Z/\chi^2$	P value
Age(year)		51(41, 56)	51(42, 55)	-1.47	0.143
Sex	Man	66 (65.35)	66 (65.35)	0.00	1.000
	Women	35 (34.65)	35 (34.65)		
Nationality	Han	94 (93.07)	95 (94.06)	0.08	0.774
	Minority	7 (6.93)	6 (5.94)		
Marriage	Married	94 (93.07)	95 (94.06)	0.08	0.774
	Unmarried/divorced	7 (6.93)	6 (5.94)		
Occupation	Farmer	13 (12.87)	10 (9.90)	13.34	0.010
	Worker	25(24.75)	49 (58.51)		
	Self-employer	4 (3.96)	5 (4.95)		
	Unemployed	44 (43.56)	28 (27.72)		
	Other	15 (14.85)	9 (8.91)		
Smoking	No	79 (78.22)	73 (72.28)	0.96	0.328
	Yes	22 (21.78)	28 (27.72)		
Drinking	No	87 (86.14)	85 (84.16)	0.16	0.692
	Yes	14 (13.86)	16 (15.84)		
SBP (mmHg)		143.85 ± 25.72	137.19 ± 22.45	1.76	0.082
DBP (mmHg)		85.16 ± 15.49	81.93 ± 14.70	1.43	0.157
TG (mmol/L)		2.85 (1.91, 6.68)	1.42 (0.85, 2.66)	4.45	<0.001
TC (mmol/L)		5.30 (4.60, 6.30)	4.55 (3.99, 5.62)	3.48	<0.001
HDL-C (mmol/L)		1.34 (1.17, 1.65)	1.36 (1.15, 1.59)	0.56	0.573
LDL-C (mmol/L)		3.12 (2.60, 3.90)	2.79 (2.40, 3.50)	2.25	0.024
AST (IU/L)		19.00 (15.00, 26.50)	20.00 (17.00, 30.50)	1.91	0.056
ALT (IU/L)		23.00 (14.00, 42.25)	19.00 (14.00, 34.00)	0.50	0.620

SDP: systolic blood pressure; DBP: systolic blood pressure; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; TG: triglycerides; TC, total cholesterol; AST: aspartate transaminase; ALT: alanine transaminase.

Statistical analysis

All the statistical analyses were performed using SAS 9.4 and R language 4.0.0. Count with percentages, mean ± standard deviation (SD) value, or median with interquartile range were used to summarize variables. Paired t-test, paired Wilcoxon test and paired chi-square test were used to explore the significance of differences among two groups. Unconditional multivariate logistic regression analysis was used to investigate the association between ncRNAs expression levels and T2DM. The diagnostic value of the RNAs was evaluated using the receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC). Spearman's correlations were performed to assess the relationship between ncRNAs and clinical parameters. P values < 0.05 or < 0.001 were considered to reflect statistical significance.

Results

Characteristics of the study subjects

A total of 101 pairs of subjects were included in this study. The median age and quartile of the diabetes group were 51 (41, 56) years, and the median and quartile age of the control group were 51 (42, 55) years. The age, smoking, drinking, sex, nationality, and marriage were not statistically different between the two groups. However, TG ($Z=4.45$, $P<0.001$), TC ($Z=3.48$, $P<0.001$) and LDL-C ($Z=2.25$, $P=0.024$) were significant differences between diabetes and the control group (Table 2).

Analysis of relative expression of target circRNA

The four screened circRNAs, including hsa_circ_0071106, hsa_circ_0000284, hsa_circ_0071271, and hsa_circ_0003344, were verified by the Wilcoxon test. The results are shown in Table 3. The hsa_circ_0071106 ($P=0.004$), hsa_circ_0000284 ($P=0.007$), and hsa_circ_0000284 ($P=0.049$) showed significantly higher expression in T2DM group than control, while the expression levels of hsa_circ_0003344 in the two groupspresented no significant differences ($P>0.05$).

Analysis of relative expression of target miRNA

To validate the four selected candidate miRNAs, including hsa-miR-29a-5p, has-miR-409-3p, hsa-miR-3690, hsa-miR-607, qRT-PCR was conducted to compare the expression levels of them. The results are shown in Table 4. Compared with the control group, the expression levels of hsa-miR-29a-5p, hsa-miR-3690, and hsa-miR-607 were significantly different upregulated($P<0.001$). But the expression level of hsa-miR-409-3p was no statistical difference between two groups($P>0.05$).

Bioinformatics prediction of hsa-miR-29a-5p, hsa-miR-607 and hsa-miR-3690-interacting lncRNAs

In order to search lncRNAs that may interact with hsa-miR-29a-5p, hsa-miR-607 and hsa-miR-3690, this study used two databases for bioinformatics prediction, and the results were shown in Figure 2. There were 28 lncRNAs in the intersection

Table 3. Verification of relative expression of target circRNA in T2DM and control group by qRT-PCR.

circRNA	Relative expression(n = 101)		*P* value
	T2DM	Control	
hsa_circ_0071106	1.176 (0.704,1.727)	1.002 (1.001,1.009)	0.004
hsa_circ_0000284	1.153 (0.698,1.693)	1.001 (1.000,1.004)	0.007
hsa_circ_0071271	1.110 (0.501,1.722)	1.012 (1.004,1.028)	0.049
hsa_circ_0003344	0.853 (0.497,1.955)	1.002 (1.001,1.008)	0.119

Table 4. Verification of relative expression of target miRNA in T2DM and control group by qRT-PCR.

miRNA	Relative expression (*n* = 101)		*P* value
	T2DM	Control	
hsa-miR-29a-5p	1.271 (0.805,1.756)	1.002 (1.001,1.008)	<0.001
has-miR-409-3p	1.140 (0.551,1.561)	1.004 (1.001,1.012)	0.223
hsa-miR-3690	1.281 (0.773,2.219)	1.003 (1.001,1.008)	<0.001
hsa-miR-607	1.313 (0.783,1.928)	1.002 (1.006,1.030)	<0.001

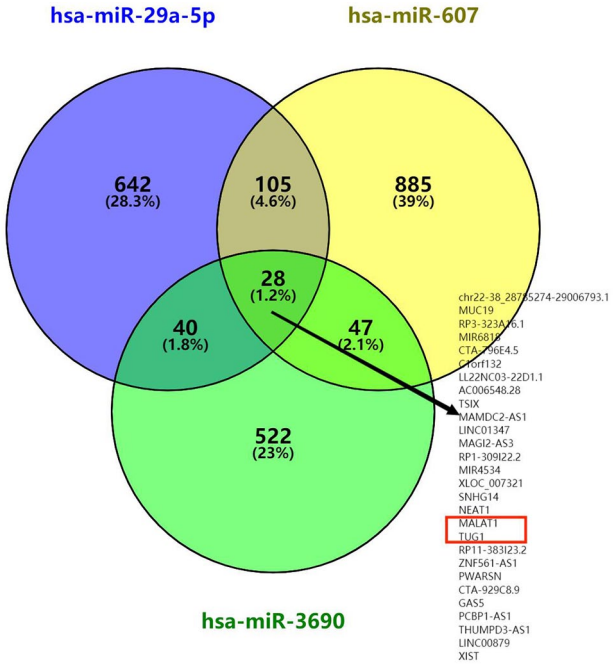

Figure 2. Venn diagrams of bioinformatics predicting lncRNAs interacting with hsa-miR-29a-5p, hsa-miR-607 and hsa-miR-3690. (A color version of this figure is available in the online journal.)

part. And combined with pieces of literature, we selected diabetes-related TUG1 and MALAT1 to enter the next step of research.[19–21] lncRNA MEG3 was found to be down-regulated in pancreatic islet cells of T2DM patients and insulin synthesis and secretion were impaired after lncRNA Meg3 inhibition in vitro.[22,23] This finding was speculated to be related to the hypermethylation of the MEG3 promoter, and its hypermethylation process was related to the miRNA-29a regulatory pathway.[24] The lncRNA MEG3 was indeed included in the lncRNAs that may interact with miR-29a-5p in the Diana-lncRNABase database. So this study will also explore the diagnostic value of lncRNA MEG3 in T2DM.

Analysis of relative expression of target lncRNA

To confirm the expression of lncRNA MEG3, lncRNA TUG1 and lncRNA MALAT1 in T2DM, this study performed relative expression analysis on 101 pairs of samples collected. The results are shown in Table 5. Significant differences of lncRNA MEG3 and lncRNA TUG1 expression levels were observed between T2DM and control group ($P < 0.001$).

Multivariate stepwise logistic regression to analysis the relation between target ncRNAs expression and T2DM

The non-conditional stepwise multivariate logistic regression model was used to identify factors that may affect T2DM.For each additional unit of the hsa_circ_0071106 expression, the risk of T2DM increases 161.2%(35.7%-534.0%), others see in Table 6.

ROC curve analysis of target ncRNAs with differential expression

The relative expressions of hsa_circ_0071106, hsa_circ_0071271, hsa_circ_0000284, hsa-miR-29a-5p, hsa-iR-607, hsa-miR-3690, lncRNA MEG3 and lncRNA TUG1 were significantly different between T2DM group and healthy control group.To determine the diagnostic values of them for T2DM, ROC curve analysis was performed and shown in Figure 3. The AUCs of lncRNA MEG3 and lncRNA TUG1 for the diagnosis of T2DM were 0.588 ([0.493–0.683], $P = 0.015$) and 0.642 ([0.548–0.736], $P < 0.001$), respectively. The AUCs of hsa-miR-29a-5p, hsa-miR-607 and hsa-miR-3690 for the diagnosis of T2DM were 0.574 ([0.477–0.671], $P = 0.034$), 0.645 ([0.553–0.737], $P < 0.001$) and 0.621 ([0.526–0.715], $P = 0.002$), respectively. The AUCs of hsa_circ_0071106, hsa_circ_0000284, and hsa_circ_0071271 for the diagnosis of T2DM were 0.563 ([0.467–0.660], $P = 0.060$), 0.554 ([0.456–0.651], $P = 0.094$) and 0.526 ([0.430–0.623], $P = 0.259$), respectively. The results showed that hsa-miR-29a-5p, hsa-miR-607, hsa-miR-3690,

Table 5. Verification of relative expression of target lncRNA in T2DM and control group by qRT-PCR.

lncRNA	Relative expression ($n=101$)		*P* value
	T2DM	Control	
lncRNA TUG1	1.315 (0.746,2.174)	1.002 (1.001,1.004)	<0.001
lncRNA MEG3	1.381 (0.627,2.621)	1.008 (1.003,1.027)	<0.001
lncRNA MALAT1	1.074 (0.570,1.857)	1.012 (1.004,1.026)	0.116

Table 6. Stepwise multivariate logistic regression of ncRNAs and T2DM.

	Estimate	Std. Error	z value	*P*(>|z|)	OR (95%CI)
hsa_circ_0071106	0.9600	0.3913	2.453	0.014	2.612 (1.357,6.340)
lncRNA TUG1	1.0301	0.3270	3.150	0.002	2.804 (1.592,5.792)
hsa-miR-607	0.9170	0.4348	2.109	0.035	2.502 (1.133,6.516)
hsa-miR-3690	0.7570	0.3913	1.934	0.053	2.132 (1.075,4.941)
hsa_circ_0071271	0.4342	0.2931	1.481	0.138	1.524 (1.050,3.200)

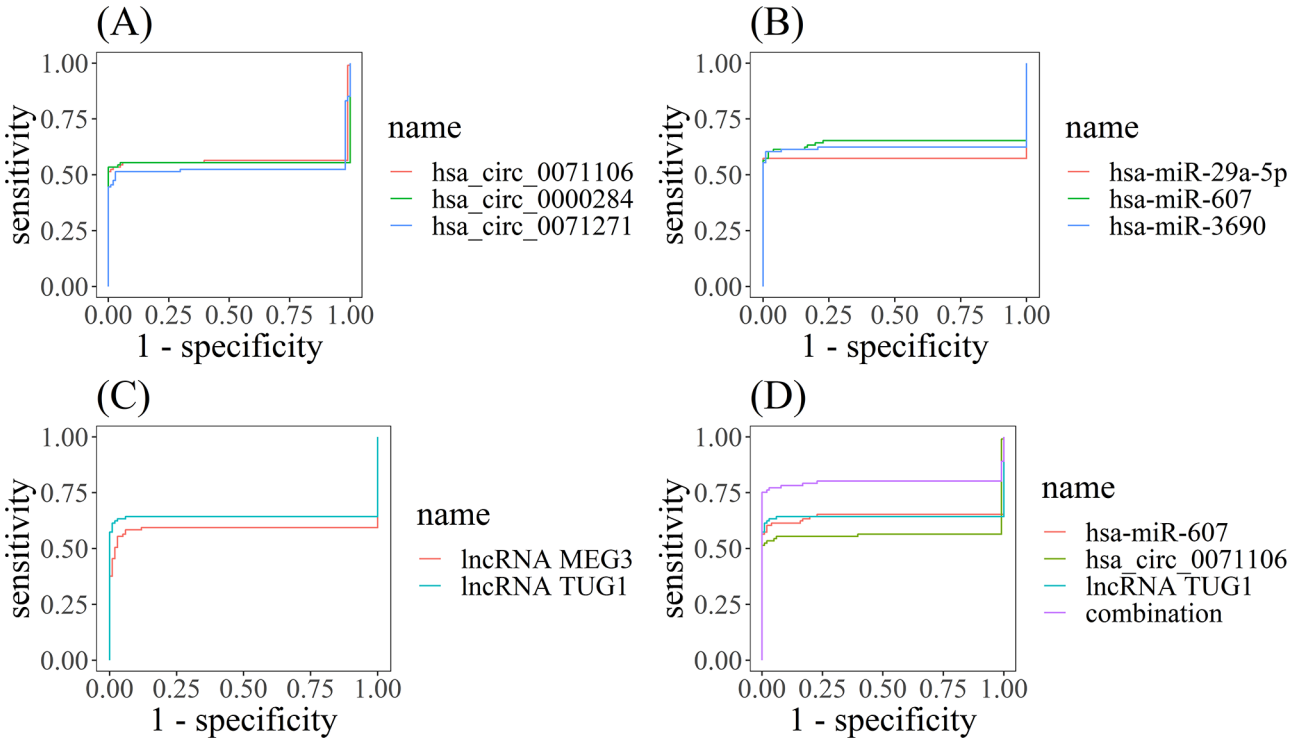

Figure 3. The ROC curve of ncRNAs in the diagnosis of T2DM. (A) The ROC curve of hsa_circ_0071106 hsa_circ_0071271, hsa_circ_0000284 (B) The ROC curve of hsa-miR-29a-5p, hsa-miR-607, hsa-miR-3690 (C) The ROC curve of lncRNA MEG3 and lncRNA TUG1 (D)The ROC curve of the combination of hsa-miR-607, lncRNA TUG1 and hsa_circ_0071106 in the diagnosis of T2DM. (A color version of this figure is available in the online journal.)

lncRNA MEG3 and lncRNA TUG1 have diagnostic value in diagnosis of T2DM.

Multivariate analysis showed that hsa_circ_0071106, hsa-miR-607, and lncRNA TUG1 are relatively closely related to T2DM. In the area under the ROC curve, we combined hsa-miR-29a-5p, hsa-miR-607, hsa-miR-3690, lncRNA MEG3, lncRNA TUG1 and hsa_circ_0071106. Among them, the combined diagnosis of hsa-miR-607, lncRNA TUG1 and hsa_circ_0071106 is the best, and the AUC of the combined effect of hsa-miR-607, lncRNA TUG1 and hsa_circ_0071106 was 0.798 ([0.720~0.875], $P<0.001$). Furthermore, the AUC was still 0.784, even after adjusting for confounding factors. The sensitivity is 74.9%, and the specificity is 98.0%. The sensitivity and specificity are shown in Table 7.

The relationship between ncRNAs expression and clinical parameters

The Spearman's correlation analysis reveals that the expression levels of hsa-miR-607, hsa-miR-3690, lncRNA MEG3 and lncRNA TUG1 are significantly positively correlated with FBG ($r_s=0.23$, $r_s=0.16$, $r_s=0.19$, $r_s=0.23$, respectively, $P<0.05$). Furthermore, hsa-miR-29a-5p and hsa-miR-607

Table 7. ROC curve of the selected ncRNAs by Q-PCR in T2DM.

Gene	AUC	95%CI	Sensitivity	Specificity	Cut-off point	Youden index	*P* value
lncRNA MEG3	0.588	0.493~0.683	0.584	0.941	1.146	0.525	0.015
lncRNA TUG1	0.642	0.548~0.736	0.634	0.970	1.045	0.604	<0.001
hsa-miR-29a-5p	0.574	0.477~0.671	0.574	1.000	1.086	0.574	0.034
hsa-miR-607	0.645	0.553~0.737	0.604	0.980	1.132	0.584	<0.001
hsa-miR-3690	0.621	0.526~0.715	0.604	0.990	1.042	0.594	0.002
hsa_circ_0071106	0.563	0.467~0.660	0.535	0.980	1.070	0.515	0.060
hsa_circ_0000284	0.554	0.456~0.651	0.535	1.000	1.047	0.535	0.094
hsa_circ_0071271	0.526	0.430~0.623	0.515	0.970	1.105	0.485	0.259
combination*	0.798	0.720~0.875	0.752	1.000	0.411	0.752	<0.001
combination**	0.784	0.686~0.864	0.749	0.980	—	0.729	<0.001

*indicates the combination of hsa-miR-607, lncRNA TUG1 and hsa_circ_0071106.
**indicates the combination of hsa-miR-607, lncRNA TUG1 and hsa_circ_0071106 after adjusting for occupation, TG and TC.TG, Triglycerides; TC, Total cholesterol.

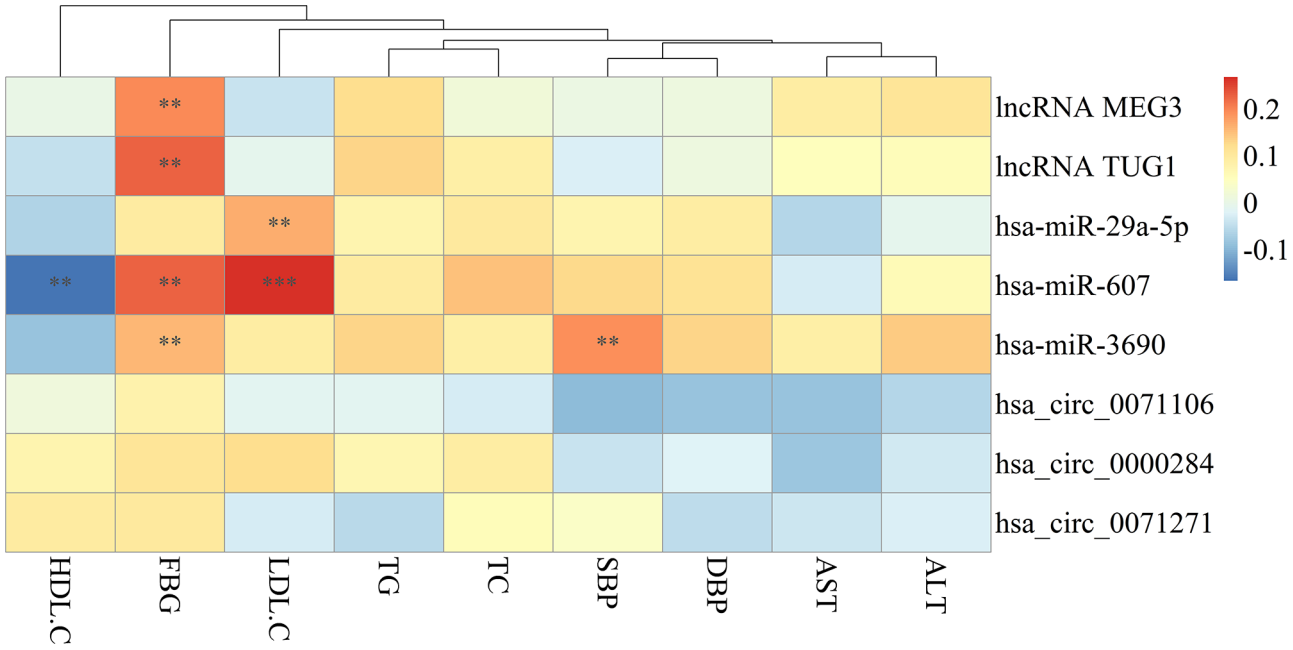

Figure 4. Correlation between differentially expressed RNAs in peripheral blood and biochemical indexes.
$^{**}P<0.05$; $^{***}P<0.001$. HDL.C, High density lipoprotein cholesterol; FBG, Fasting blood glucose; LDL.C, Low density lipoprotein cholesterol; TG, Triglycerides; TC, Total cholesterol; SBP, Systolic blood pressure; DBP, Systolic blood pressure; AST, Aspartate transaminase; ALT, Alanine transaminase. (A color version of this figure is available in the online journal.)

are positively correlated with LDL-C ($r_s = 0.17$, $r_s = 0.27$, respectively, $P < 0.05$). More details are mentioned in Figures 4 and 5. It implies that hsa-miR-3690, hsa-miR-29a-5 and hsa-miR-607, lncRNA MEG3 and lncRNA TUG1 are related to clinical indicators of T2DM.

Discussion

With the development of economy and the increasing aging population, T2DM has become one of the diseases that have a serious impact on human health. It is not only difficult to cure but also has a huge impact on the quality of life of patients, which is a major problem facing the world. Despite the clinical diagnosis of T2DM continue to improve and optimize, each clinical diagnostic method has its limitations, including different sensitivity in different populations. An earlier study found that more people were diagnosed by HbA1c than by FPG only in older age groups.[25] Moreover, in order to obtain accurate index values, it is often necessary to repeat measurements. So these diagnostic methods are often combined with clinical symptoms in practical clinical applications. ncRNA has the characteristics of tissue specificity, disease specificity, timing specificity, high stability, and post transcriptional regulation effect. These features contribute to the emergence of ncRNAs as novel biomarkers for clinical diagnosis.[26]

Our data indicated that hsa_circ_0071106, lncRNA TUG1 and hsa-miR-607 is an effective method for diagnosing of T2DM. The AUC was 0.798, the sensitivity was 0.752, and the specificity was 1.000. Our results are biologically plausible for the following reasons.

First, early stage of T2DM onset is invisible. T2DM is characterized by a gradual development of hyperglycemia, and an asymptomatic and highly variable prodromic phase,

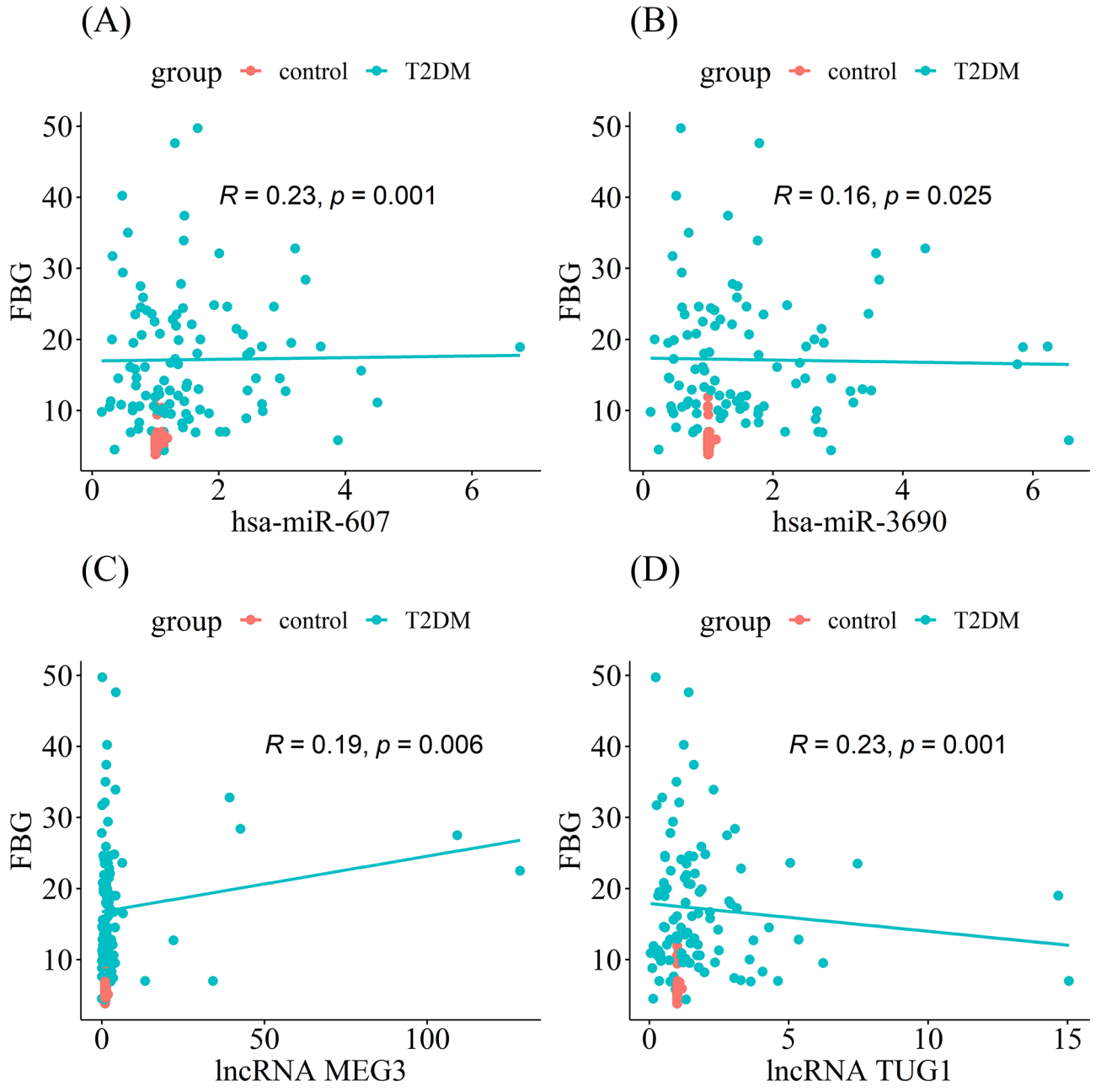

Figure 5. Correlation between differentially expressed ncRNAs in peripheral blood and FBG. (A) Correlation of expression of the hsa-miR-607 in Peripheral Blood and FBG. (B) Correlation of expression of the hsa-miR-3690 in Peripheral Blood and FBG. (C) Correlation of expression of the lncRNA MEG3 in Peripheral Blood and FBG. (D) Correlation of expression of the lncRNA TUG1 in Peripheral Blood and FBG. FBG, Fasting blood glucose. (A color version of this figure is available in the online journal.)

which is not severe enough for the patient to note any of the classic symptoms of diabetes in its earlier stages.[27] For this reason, the tools used currently to screen and diagnose T2DM and to detect individuals with prediabetes do not adequately predict the onset of the disease and monitor its progression.[28] Data from China showed that the undiagnosed rate of T2DM was 63.3% in 2013.[2] A cohort study had demonstrated the miR-150 and miR-30a-5p were deregulated in plasma several years before the diagnosis of T2DM, as the expression of them gradually increased from normoglycemia to prediabetes up to T2DM.[28] It suggests aberrantly expressed ncRNAs are tools for T2DM prediction, prevention and diagnosis in individuals. Therefore, their clinical application is highly envisaged.

Second, hsa_circ_0071106, hsa-miR-607, and lncRNA TUG1 are associated with the development of T2DM, respectively. From the experimental results of this study, in consistent with other studies, increased expression of hsa_circ_0071106, miR-607 and lncRNA TUG1 could increase the risk of developing T2DM, respectively. They were not only differentially expressed, but also had the highest diagnostic value among the differentially expressed lncRNAs, circRNAs, and miRNAs studied, respectively. In the aspect of T2DM pathogenesis, more and more evidences demonstrated that hsa_circ_0071106, hsa-miR-607 and lncRNA TUG1 are involved in the development of T2DM. We predicted the function of hsa_circ_0071106 in the early research. The function prediction results showed that hsa_circ_0071106 was

involved in MAPK signaling pathway that regulates insulin signal transduction after the enrichment of the KEGG pathway.[7] Jiang *et al*.[29] found miR-607 is judged as a potential biomarker for patients with pancreatic ductal carcinoma, indicating that miR-607 plays a vital role in pancreatic islet tissue. We found that lncRNA TUG1 increase the risk of T2DM. Similar data were found in diabetic atherosclerosis patients, suggesting that overexpressed lncRNA TUG1 promotes the occurrence of diabetes complications.[30] In vivo and in vitro experiments, it had shown that knocking out lncRNA TUG1 can promote cell apoptosis and impair the function of β cells, and its expression is dynamically regulated by glucose.[19] Therefore, it is feasible to envisage hsa_circ_0071106, miR-607 and lncRNA TUG1 as biomarkers for T2DM.

Third, ROC analysis showed hsa_circ_0071106, hsa-miR-607 and lncRNA TUG1 combine had a high accuracy for discriminating T2DM from healthy control, suggesting that combined diagnosis value is better than a single ncRNA. It makes plausible sense in terms of the intricate and complex pathogenesis of T2DM. A growing evidence that circRNA, lncRNA, and miRNA interactions together are involved in regulating T2DM, such as inhibition of hsa_circ_0054633 protected against high glucose-induced NES2Y cell apoptosis and impairment of insulin secretion by regulating miR-409-3p/caspase-8 axis.[16] Thus, a method based on a combination of multiple biological indicators can better ensure the accuracy of diagnosis which is also the current trend of applying ncRNAs as a diagnostic method. Although their interactions were not investigated in depth in this study, we established the circRNA-miRNA-lncRNA network by database prediction is scientific. And in multifactorial analysis, the results were consistent with what we envisaged. The results showed that the increased expression of has_ circ_0071106, hsa-miR-607 and lncRNA TUG1 were significant predictors of T2DM. The results of another study are consistent with ours, they found that combining two circRNAs is better in diagnosing T2DM than using one circRNA data alone.[31]

In addition, we found that there were 4 ncRNAs which were positively correlated with FBG, suggesting that the expression level of ncRNAs can reflect the blood glucose concentration. The expression of lncRNA MEG3 and lncRNA TUG1 in diabetic patients is related to blood glucose concentration. We could found similar results in other studies. Such as Sathishkumar and his colleagues found that majority of the altered lncRNAs were positively correlated with poor glycemic control, insulin resistance, transcriptional markers of senescence, inflammation.[32] This observation was further supported by evidence showing that ncRNAs as biomarkers of T2DM is credibility and reliability, especially hsa-miR-607 and lncRNA TUG1.

Furthermore, hsa-miR-29a-5p and hsa-miR-607 were positively correlated with the concentration of LDL-C. This indicates that in the peripheral blood of diabetic patients, the level of hsa-miR-607 and hsa-miR-29a-5p would increase the concentration of LDL-C. These above findings suggest that the selected ncRNAs will affect diabetes related clinical indicators.

However, there were many limitations in our study. First, this study is only a case-control study, and there are deficiencies in causality. However, our cases and controls are from the same village, with the same gender and similar age, which increases the comparability. Second, although it ignored the risk factors of BMI in the T2DM population, TC, TG and LDL-C were considered in the study.Third, only the peripheral blood qRT-PCR was used to verify the relative expression of each molecule. It is necessary to further verify the interaction of miRNA, circRNA and lncRNA through experiments such as fluorescence resonance energy transfer and luciferase, and to further verify its function through experiments such as over expression.

Conclusions

In summary, this study combined epidemiological and bioinformatics methods to explore the relationship between miRNA, circRNA and lncRNA and T2DM, and found hsa-miR-29a-5p, hsa-miR-607, hsa-miR-3690, lncRNA MEG3, lncRNA TUG1 and hsa_circ_0071106 are associated with the risk of T2DM. This study confirmed that the diagnostic value of the combination of hsa-miR-607, lncRNA TUG1 and hsa_circ_0071106 is higher than that of a single index, which provides a theoretical basis for studying the network mechanism of T2DM and provides an effective diagnostic target for the diagnosis of T2DM.

AUTHORS' CONTRIBUTIONS

M.N.S. and T.X.Y. anlalyzed the data and drafted the manuscript. Y.J.Y. collected the samples. Y.Y.Z. and Q.T.C. had done the experiments. R.F.L. and Z.P.Z. designed the research and drafted the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The Ethics Committee of Guangdong Pharmaceutical University approved this study and all participants provided written informed consent.

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