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Article

Hsa_circ_0054633 in Peripheral Blood Can be Used as a Diagnostic Biomarker of Pre-Diabetes and Type 2 Diabetes Mellitus

Muwei Li [1,*], Zhenzhou Zhao [1,†], Xuejie Li [1,†], Chuanyu Gao [1], Dongdong Jian [2], Peiyuan Hao [1] and Lixin Rao [1]

[1]Department of Cardiology, People's Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou 450003, China

[2]Department of Cardiology, the First Affiliated Hospital of Zhejiang University, Zhejiang University Hangzhou 310003, China

*Correspondence: limuwei@medmail.com.cn

†These authors contributed equally to this work.

Abstract: The purpose of current study was to investigate the expression characteristic of circular RNAs (circRNAs) in peripheral blood of type 2 diabetes mellitus (T2DM) patients and their potentials as diagnostic biomarkers for pre-diabetes and T2DM. In present study, the circRNAs in the peripheral blood from 6 healthy individuals and 6 T2DM patients were collected for microarray analysis. The results indicated that there were 489 differentially expressed circRNAs, of which 78 were upregulated and 411 were downregulated in the T2DM group. Then we selected 5 circRNAs as the candidate biomarkers under a stricter screening criteria and further verified them in another cohort (control group, n=20; pre-diabetes group, n =20; T2DM group; n=20). 3 of the 5 circRNAs presented upregulated expression in the experimental groups, including 2 circRNAs of the T2DM group that had higher expression than the pre-diabetes group. Hsa_circ_0054633 was identified to have the largest area value under the carve (AUC). In another independent cohort (control group, n=60; pre-diabetes group, n=63; T2DM group, n=64), the diagnostic capacity of hsa_circ_0054633 was tested. The results showed that the AUC for the diagnosis of pre-diabetes was 0.751(95% confidence interval=[0. 666-0.835], $P<$ 0.001) while it was 0.793 ([0.716-0.871], $P<0.001$) for the diagnosis of T2DM. After including the risk factors of T2DM, the AUC increased to 0.841 ([0.773-0.910], P <0.001) and 0.834 ([0.762-0.905], P <0.001), respectively. Hsa_circ_0054633 presented a certain diagnostic capability for pre-diabetes and T2DM.

Keywords: circular RNAs (circRNAs); circulating circRNA; type 2 diabetes mellitus (T2DM); pre-diabetes; microarray analysis; biomarker

1. Introduction

According to the international diabetes federation (IDF) Diabetes Atlas (7th edition, 2015), there were nearly 410 million diabetic patients worldwide, 46.5% of which haven't been diagnosed currently. By 2040, the number of patients with diabetes may increase to 642 million[1]. There are no obvious clinical symptoms at the early stage of T2DM, and the present diagnostic methods show variously insufficient in early diagnosis of T2DM. Patients often go to the hospital for diagnosis and treatment when they have obvious

 doi:10.20944/preprints201608.0225.v1

clinical symptoms, which mean the optimal treatment window was missed. Therefore, a new highly sensitive biomarker will be of great value.

In recent years, with the advancements in genomics, such as the technological renovations in genome-wide association studies (GWAS) and the implementation of encyclopedia of DNA elements (ENCODE), the single nucleotide polymorphism (SNP) sites of related encoding sequences of some complicated diseases including T2DM have been dug out gradually[2-4]. Researchers have found that human genome can be widely transcribed into a large number of non-coding RNAs which are closely linked to the occurrence and development of diseases[5]. CircRNAs are a type of closed circular non-coding RNAs, formed by exon, intron, or reverse splicing of the two [6, 7]. Because lacking of 5'-cap and 3'-poly (A) tail in the circular structure, circRNAs cannot be recognized and hydrolyzed by RNA exonuclease. Therefore, circRNAs have a higher biological stability than most of the linear RNAs [8]. CircRNAs have multiple regulatory mechanisms of gene expression[9]: some circRNAs can be used as "miRNA sponges", playing the role of posttranscriptional regulation by competitive combination with miRNA[10], circRNAs can also regulate transcription by interacting with snRNA or RNA polymerase Ⅱ in the nucleus[11], it also can competitively regulate RNA splicing by binding to transcription factors[12]. Substantial amount of circRNAs are widely distributed in the cytoplasm and nucleus, and a variety of body fluids such as saliva and serum exosomes [13, 14]. Thus, we hypothesized that circRNAs in the peripheral blood could participate in some kinds of biological processes.

CircRNAs play important roles in various diseases, including tumor, atherosclerosis, osteoarthritis, pulmonary fibrosis, myotonic dystrophy and Alzheimer's disease [15-18]. The high biological stability of circRNAs is the precondition for its usage as biomarkers for various diseases: Li and Chen et al. found that the expression level of Has_circ_002059 was significantly higher in non-cancer tissues than that of gastric cancer tissues, and it has been used as a new biomarker for gastric cancer [19]. Li and Zhang et al. found that circ-ITCH could be used for clinical diagnosis of esophageal cancer [20]. The study of Qin and Liu et al. discovered that hsa_circ_0005075 could be used as a potential biomarker for hepatocellular carcinoma [21]. For T2DM, CDR1as have been found to affect the insulin secretion and β cell renewal [22]. Therefore, it could be speculated that some circRNAs in peripheral blood could be used as diagnostic biomarkers for pre-diabetes and T2DM. In this study, we compared circRNAs expression profile in peripheral blood of T2DM patients and matched control subjects by microarray analysis followed by verifying our findings in larger independent cohorts. In the end, it was found that hsa_circ_0054633 was a sensitive and specific biomarker for pre-diabetes and T2DM diagnosis.

2. Material and Methods

2.1. Study population.

In this study, a total of 259 individuals were classified into 3 cohorts (clinical and demographic characteristics are showed in Table 1-3). The participants of experimental groups were enrolled from the outpatients and inpatients of department of cardiology and department of endocrinology from July 2015 to June 2016, while control group participants were included from department of physical examination. Subjects with the following

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situations were excluded: 1, malignancies; 2, liver and kidney dysfunction; 3, any other clinically systemic acute or chronic inflammatory diseases; 4, autoimmune disease; 5, untreated hypertension; 6, any other endocrine diseases, except for T2DM. This experiment has been approved by the Ethics Committee of our hospital. All the subjects signed the informed consent. This study protocol conformed to the ethical guidelines of 1975 Declaration of Helsinki.

Table 1. The clinical and demographic characteristics of the first cohort.

	Control group (n=6)	T2DM group （n=6）	*P* value
Male gender	3（50%）	3（50%）	1
Age	60±2.3	62.3±6.2	0.42
Hypertension	1（16.7%）	2（33.3%）	1
Smoker	1（16.7%）	1（16.7%）	1
BMI(kg/m^2)	23.3±2.1	24.7±1.2	0.178
TC(mmol/L)	3.5±0.9	3.9±1.1	0.506
TG(mmol/L)	0.9±0.2	1.4±0.6	0.073
HDL(mmol/L)	1.3±0.3	1.1±0.2	0.202
LDL(mmol/L)	1.8±0.7	2.1±0.9	0.559
ALT(U/L)	36.3±17.8	33.7±17.9	0.801
AST(U/L)	23.5±3.8	19.8±7.3	0.299
Scr(μmol/L)	58.7±3.9	60.0±10.7	0.781
FT4I(pmol/L)	14.4±2.7	14.1±1.9	0.801
HbA1c(%)	5.4±0.1	6.9±0.7	0.003
GLU(mmol/L)	5.0±0.4	9.8±2.0	<0.001
OGTT 2h(mmol/L)	7.0±0.4	13.8±1.3	<0.001

Table 2. The clinical and demographic characteristics of the second cohort.

	Control group (n=20)	Pre-diabetes group (n=20)	T2DM group (n=20)	*P* value
Male gender	8(40.0%)	11(55.0%)	12(60.0%)	0.42
Age	49.6±4.7	47.2±5.9	51.1±5.5	0.083
Hypertension	5(25.0%)	6 (30.0%)	8 (40.0%)	0.583

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Smoker	3(15.0%)	5(25.0%)	5(25.0%)	0.675
BMI(kg/m²)	23.6±2.1	22.8±2.2	23.7±2.0	0.343
TC(mmol/L)	3.7±0.8	3.9±1.0	4.2±0.9	0.196
TG(mmol/L)	1.5±0.4	1.6±0.5	1.7±0.7	0.342
HDL(mmol/L)	1.3±0.5	1.1±0.2	1.1±0.3	0.501
LDL(mmol/L)	2.5±0.6	2.8±0.6	2.8±0.6	0.083
ALT(U/L)	24.8±6.6	24.1±2.9	27.7±7.6	0.415
AST(U/L)	24.9±5.6	26.1±4.4	23.2±4.5	0.169
Scr(μmol/L)	59.2±6.5	58.3±8.0	61.2±4.9	0.387
FT4I(pmol/L)	14.3±1.6	15.2±2.4	13.9±1.9	0.111
HbA1c(%)	5.0±0.2	6.0±0.2	7.5±0.7	＜0.001
GLU(mmol/L)	5.0±0.4	6.5±0.2	7.9±0.6	＜0.001
OGTT 2h(mmol/L)	6.8±0.5	8.9±1.4	12.9±1.0	＜0.001

Table 3. The clinical and demographic characteristics of the third cohort.

	Control group (n=60)	**Pre-diabetes group (n=63)**	**T2DM group (n=64)**	***P* value**
Male gender	29(48.3%)	32(50.8%)	35(54.7%)	0.774
Age	49.0±5.5	49.0±6.6	50.6±5.7	0.222
Hypertension	21(35.0%)	25(39.7%)	31（48.4%）	0.302
Smoker	12(20.0%)	13(20.6%)	18(28.1%)	0.484
BMI(kg/m²)	24.5±3.8	25.0±3.1	23.9±3.8	0.192
TC(mmol/L)	3.6±0.7	4.1±0.8	4.3±0.5	＜0.001
TG(mmol/L)	1.4±0.4	1.6±0.3	1.5±0.4	0.038
HDL(mmol/L)	1.0±0.2	1.1±0.3	1.0±0.2	0.362
LDL(mmol/L)	2.5±0.8	2.6±0.6	2.5±0.4	0.553
ALT(U/L)	25.5±8.4	26.5±4.8	26.3±5.9	0.648
AST(U/L)	23.2±5.6	24.8±3.1	24.5±4.6	0.156
Scr(μmol/L)	58.0±8.1	59.8±6.1	59.6±6.0	0.27
FT4I(pmol/L)	14.4±1.6	15.0±2.5	14.9±2.2	0.212

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HbA1c(%)	5.0±0.4	5.9±0.3	7.5±0.6	<0.001
GLU(mmol/L)	4.8±0.5	6.2±0.3	8.2±1.0	<0.001
OGTT 2h(mmol/L)	6.6±0.4	8.6±0.9	14.4±1.8	<0.001

BMI: body mass index; TC: total cholesterol; TG: triglyceride; HDL: high density lipoprotein; LDL: low density lipoprotein; ALT: alanine transaminase; AST: aspartate transaminase; Scr: serum creatinine, FT4I: free thyroxine index; GLU: glucose; OGTT: oral glucose tolerance test.

2.2. Study Process

The process of this study is showed in Figure 1. Each subject in this study was tested by oral glucose tolerance test (OGTT) to determine if one was healthy or with pre-diabetes or T2DM. 6 control individuals and 6 T2DM patients with matched clinical features were selected to collect venous blood samples, and the total RNAs were extracted for microarray analysis. Then the screened circRNAs were validated in an independent cohort (control group, n=20; pre-diabetes group, n=20; T2DM group, n=20). After receiver operating characteristic (ROC) curve analysis, the circRNA with the best diagnostic value was selected as the biomarker, and its diagnostic value was validated in a cohort with 187 subjects (control group, n=60; pre-diabetes group, n=63; T2DM group, n=64).

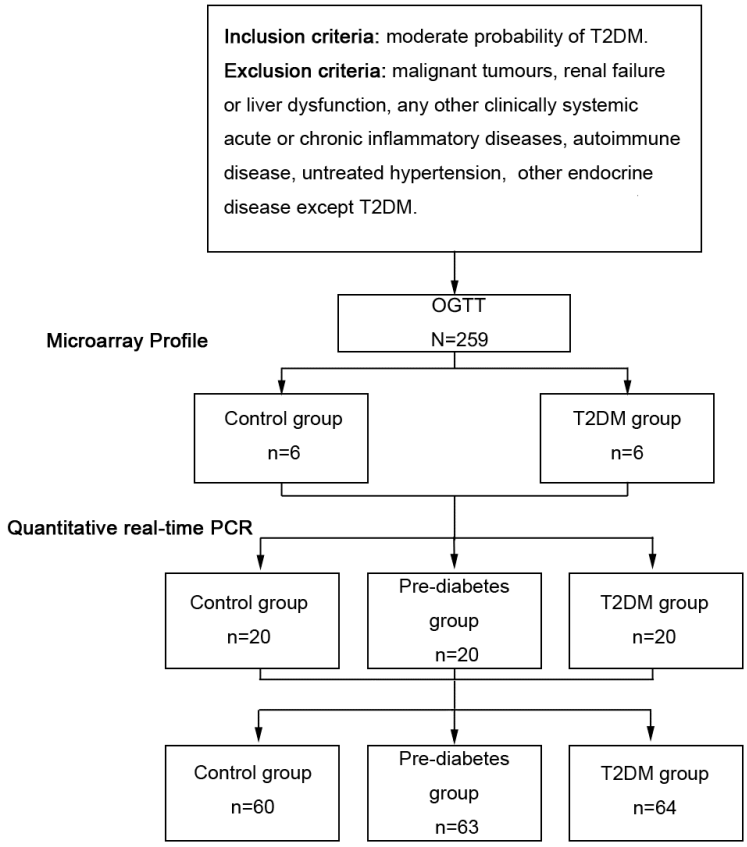

Figure 1. Study flow. OGTT: oral glucose tolerance test; PCR: polymerase chain reaction.

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2.3. The definitions of pre-diabetes and T2DM and collection of whole blood sample

In this study, pre-diabetes and T2DM were diagnosed according to the 1998 Standards by World Health Organization (WHO) [23], meeting any one of the following criteria could be diagnosed as T2DM: 1. fasting plasma glucose (FPG) ≥ 125mg/dL (7.0mmol/L), fasting is defined as no caloric intake for at least 8 hours; 2. 2-hours post-load plasma glucose ≥ 200mg/dL (11.1mmol/L) during the OGTT.

With any of the following standards, the patient was diagnosed with pre-diabetes: 1. FPG: ≥ 110mg/dL (6.1mmol/L) and < 125 mg/dL (7.0mmol/L); 2. 2-hours post-load plasma glucose ≥ 140mg/dL (7.8mmol/L) and < 200mg/dL (11.1mmol/L) during an OGTT.

Blood sample collection: after overnight fasting (no food intake for at least 8-10 hours), 2ml blood was collected from the median cubital vein of each patient before breakfast, and then stored in an EDTA (ethylene diamine tetraacetic acid) anticoagulant vacutainer. Then the total RNA was extracted as soon as possible.

2.4. RNA Extraction and Quantitative RT-PCR

Within 20 minutes after blood collection (the sample was stored in 4 ℃ ice box during this time interval), fast total RNA extraction kit (centrifugal column type) (Biotech, Beijing, China) was used to extract total RNA from 1ml whole blood according to the manufacturer's instruction. RNA was then dissolved in RNase-Free water, and its yield and purity were measured by NanoDrop 2000 (Thermo Scientific, USA). According to the manufacturer's instructions, PrimeScript RT Reagent Kit (Takara Bio, Inc., Japan) was used for the production of cDNA (complementary DNA) by reverse transcription. Q-PCR was performed using SYBR-Green Premix Ex Taq (Takara Bio, Inc., Japan) and monitored by ABI PRISM 7500 Sequence Detection System (applied Biosystems, Life Technologies, USA). The relative expression levels of circRNAs were determined via Q-PCR. The sequences of primers used in the Q-PCR assay are showed in the Table 4.

Table 4. Nucleotide sequences of primers used for Q-RCR.

	Forward	Reverse	Product length
hsa_circ_0068087	TCATTCCTCTATTTGTACAGTGGCT	GGCCCCTCAGTGTACGTCTT	141
hsa_circ_0054633	TTGCTTTCTACACTTTCAGGTGAC	GCTTTTTGTCTGTAGTCAACCACCA	110
hsa_circ_0124636	TTGCATTGTGGGCGGTATGC	TCCCCGGGTATACAAAAGTGAGA	127
hsa_circ_0139110	CCAAGCAGTCACAGAAGCTGG	ATACAGGCACCCAGGTAGGC	148
hsa_circ_0018508	TCTTTGCCACATATTGGGTGACT	ACACCAGGTACCGGTTATCCA	111
hGAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG	202

2.5. CirRNA microarray analysis

The RNAs of peripheral blood of 6 control subjects and 6 patients with T2DM were extracted for microarray analysis. The purity and concentration of RNA were determined from OD260/280 readings using spectrophotometer (NanoDrop ND-1000). The integrality

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of RNA was determined by the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The extracted RNAs were digested, dephosphorylated, denatured, amplified and labeled with Cy3-dCTP according to the
manufacturer's specifications. The purified RNAs were hybridized to a microarray (Agilent human circRNA Array V2.0) containing 170,340 probes of human circRNAs. Then the microarray data of the circRNAs were analyzed by the GeneSpring software V13.0 (Agilent). The thresholds used here were as follows: fold change: ≥ 2 or ≤ −2, $p < 0.05$ for the t- test.

2.6. Data analysis

Variables of different distributions were expressed as mean ± standard deviation, median (quartiles) or percentages when it fits. In the scatter plot for circRNAs expression, the horizontal lines represented the medians. The chi-square test was used for categorical variables; Kolmogorov-Smirnov and Shapiro-Wilk test were performed to check data normality for continuous variables, followed by the test for homogeneity of variances. The significance differences of clinical and demographic indicators were tested by one-way analysis of variance (ANOVA), if the continuous variables consistent with the normal distribution and homogeneity of variance, or by The Kruskal-Wallis H test, if not. The clinical diagnostic value of a given circRNA was verified by ROC curve analysis, AUC=0.5 meant no diagnostic value; the cut-off value and corresponding sensitivity and specificity could be identified through ROC curve analysis. Furthermore, logistic regression analysis was performed to obtain odds ratio (OR) when the relative expression of circRNAs was expanded to 10 times. $P<0.05$ was considered statistically significant. All statistical analyses were conducted by SPSS 22.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Expression profiles of circRNAs in peripheral blood of diabetic patients

In order to investigate the expression profiles of circRNAs in healthy individuals and T2DM patients, 6 subjects of the control group and 6 patients with T2DM were selected. Microarray analysis of the expression profiles of circRNAs in peripheral blood was performed by the Agilent human circRNA Array (V2.0). The results showed obvious differences in expression profiles of circRNAs between the two groups (shown in Figure 2). In the microarray analysis, a total of 489 circRNAs were found with significant expression differences between the two groups: 78 of them were upregulated, and 411 were downregulated in the T2DM group (shown in Supplementary Table S1). To make the biomarker more applicable in clinical practice, the candidate biomarkers were selected from the 78 up-regulated circRNAs, under a stricter screening criterion: fold change>2.4, $p < 0.01$. 5 circRNAs met these standards: hsa_circ_0068087, hsa_circ_0054633, hsa_circ_0124636, hsa_circ_0139110 and hsa_circ_0018508 (highlighted in Supplementary Table S1). These circRNAs were used as candidate biomarkers for later validation in a larger cohort.

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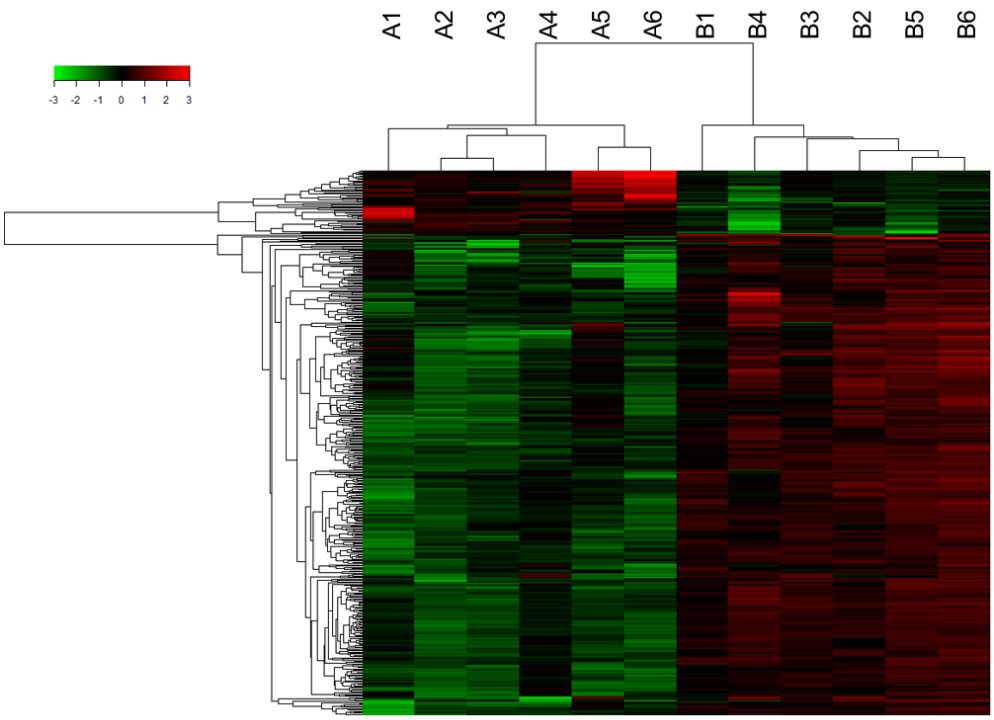

Figure 2. Heat map of circRNAs microarray profile in control individuals and T2DM patients. The expression of circRNAs is hierarchically clustered on the y axis, and blood samples are hierarchically clustered on the x axis. Expression level is presented in red and green, indicating upregulated and downregulated respectively. Numbers with A and B indicate control individuals and T2DM patients.

3.2. The expression profile of cirRNA verified by Q-PCR

For the validation of the 5 selected candidate circRNAs, Q-PCR was carried out in an independent cohort (control group, n=20; pre-diabetes group, n=20; T2DM group, n=20). The results are showed in Figure 3: the expression levels of hsa_circ_0124636 and hsa_circ_0139110 among the three groups presented no significant differences; the expression levels of hsa_circ_0018508 in pre-diabetes group and T2DM group were of no difference, while both higher than that of the control group. The expression levels of hsa_circ_0054633 and hsa_circ_0068087 were statistically significant differences among the 3 groups, with the level increasing gradually from the control group, pre-diabetes group to the T2DM group.

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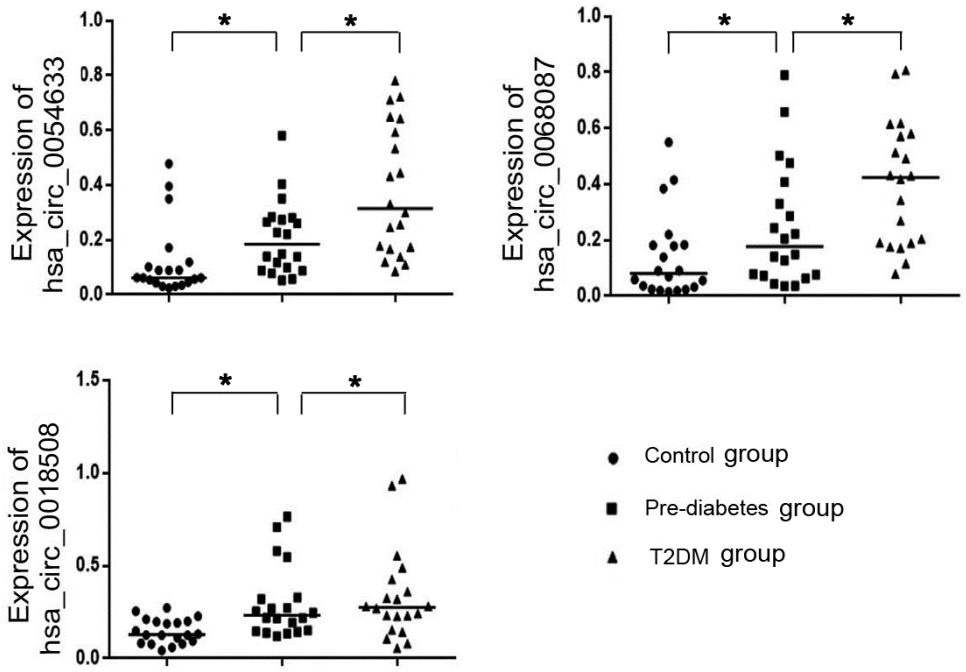

Figure 3: Expression levels of selected 3 circRNAs quantified by Q-PCR. The relative levels of circRNAs were normalized to levels of the control (hGAPDH). *: P < 0.05.

3.3. ROC curve analysis of circRNA with differential expressions

In order to judge the diagnostic values of hsa_circ_0054633 and hsa_circ_0068087 for pre-diabetes and T2DM, ROC curve analysis was performed (seen in Figure 4): the AUCs of hsa_circ_0054633 for the diagnoses of pre-diabetes and T2DM were: 0.747 ([0.589-0.906], *P*=0.007), 0.72 ([0.562-0.878], *P*=0.017), respectively. The AUCs of hsa_circ_0068087 for the diagnoses of pre-diabetes and T2DM were: 0.692 ([0.529-0.856], *P*=0.037) and 0.717 ([0.557-0.878], *P* =0.019); the cutoff values of sensitivity and specificity are showed in Table 5. Considering that hsa_circ_0054633 showed higher AUC and lower *P* values than hsa_circ_0068087, the former was chosen as the diagnostic biomarker for pre-diabetes and T2DM.

Table 5. Validation of selected circRNAs by Q-PCR.

circRNA		AUC	95%CI	*P* value	Sensitivity	Specificity	Fold change
	pre-diabetes	0.747	0.589-0.906	0.007	0.75	0.70	1.7
hsa_circ_0054633	T2DM	0.720	0.562-0.878	0.017	0.55	0.85	1.8
	pre-diabetes	0.692	0.529-0.856	0.037	0.80	0.50	1.8
hsa_circ_0068087	T2DM	0.717	0.557-0.878	0.019	0.90	0.50	1.6

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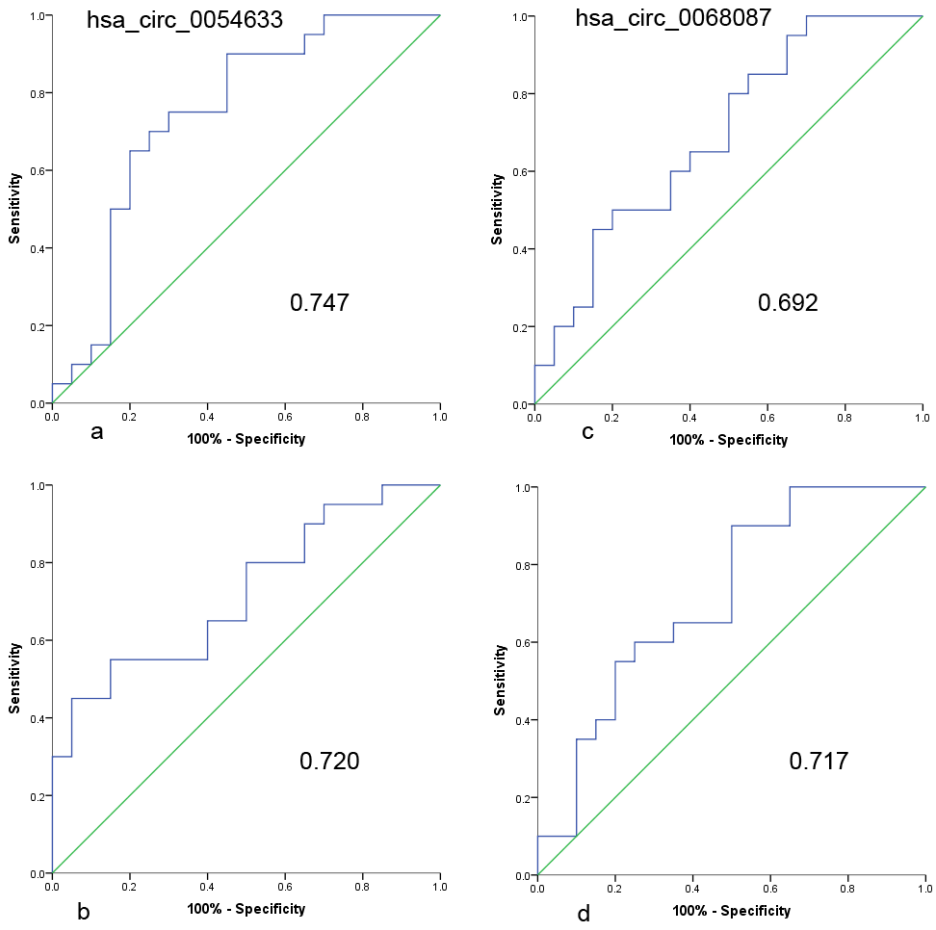

Figure 4. ROC curve analyses of has_circ_0054633 and has_circ_0068087. The AUC values were given on the graphs. a and c indicate the ROC curve analysis of hsa_circ_0054633 and has_circ_0068087 for the diagnoses of pre-diabetes; b and d indicate the ROC curve analysis of hsa_circ_0054633 and has_circ_0068087 for the diagnoses of T2DM, respectively.

3.4. Further clinical validation of the biomarker

To verify its clinical diagnostic capability, hsa_circ_0054633 was tested in another cohort with a larger sample size (control group, n=60; pre-diabetes group, n=63; T2DM group, n=64). The results were showed in Figure 5: the expression level of hsa_circ_0054633 increased gradually from the control group, pre-diabetes group to the T2DM group, with the fold change of 1.8 between the pre-diabetes group and the control group, and 1.7 between the T2DM group and the pre-diabetes group. Then the ROC curve analysis was performed (shown in Figure 6), as the biomarker for the diagnosis of pre-diabetes and T2DM, the AUCs were 0.751 ([0.666-0.835], P<0.001), 0.793 ([0.716-0.871], P<0.001), respectively, and the crude ORs were 3.05 ([1.803-5.159], P<0.001) and 2.056 ([1.530-2.762], P<0.001), respectively. After introducing risk factors of T2DM: smoking, hypertension, BMI, TC, TG, HDL and LDL, the AUCs increased to 0.841 ([0.773-0.910], P<0.001) and 0.834 ([0.762-0.905], P <0.001), while the adjusted ORs were 6.797 ([3.025-15.273], P <0.001) and 2.769 ([1.881-4.077], P <0.001), respectively (seen in Table 6).

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Table 6. Sensitivity and specificity of has_circ_0054633 and has_circ_0054633 combined with risk factors.

Diagnostic model		AUC	95% CI	sensitivity	specificity	*P* value
has_circ_0054633	pre-diabetes	0.751	0.666-0.835	0.587	0.767	<0.001
	T2DM	0.793	0.716-0.871	0.719	0.778	<0.001
has_circ_0054633+risk	pre-diabetes	0.841	0.773-0.910	0.778	0.783	<0.001
factors	T2DM	0.834	0.762-0.905	0.766	0.794	<0.001

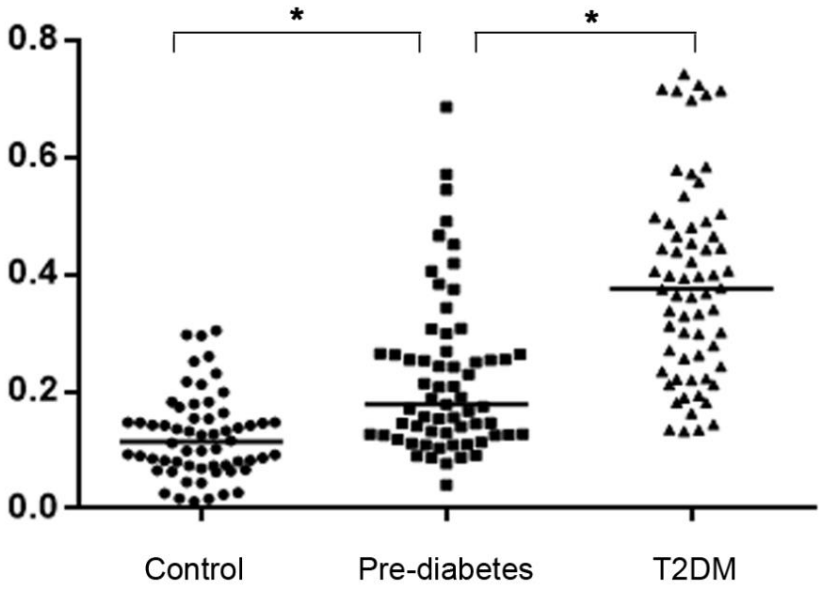

Figure 5. Expression levels of has_circ_0054633 quantified by Q-PCR.
*: P < 0.05.

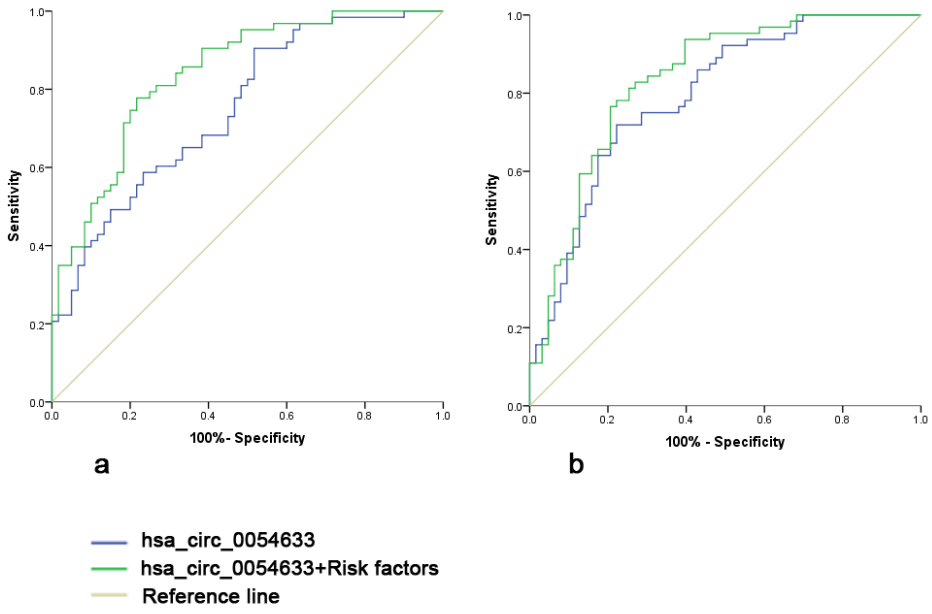

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Figure 6. ROC curve analyses of has_circ_0054633. a: ROC curve analysis for the diagnosis of pre-diabetes, b: ROC curve analysis for the diagnosis of T2DM.

3.5. Expression levels of biomarker in different gender and age groups

To investigate the expression levels of hsa_circ_0054633 in different gender and age groups, the three groups of the third cohort were divided according to age (50 years old as the division line) and gender, respectively. The results are showed in Table 7, indicating that there were no differential expression of hsa_circ_0054633 levels among different gender and age groups.

Table 7. Expression levels of hsa_circ_0054633 in different gender and age groups.

Variable	Control group			pre-diabetes group			T2DM group		
	Number	Expression quantity Median (quartile)	*P* value	Number	Expression quantity Median (quartile)	*P* value	Number	Expression quantity Median (quartile)	*P* value
age									
＜50	37	0.116 (0.072,0.148)	0.665	34	0.199 (0.128,0.277)	0.363	32	0.414 (0.235,0.496)	0.301
≥50	23	0.102 (0.073,0.199)		29	0.155 (0.120,0.259)		32	0.350 (0.246,0.450)	
gender									
male	29	0.099 (0.066,0.169)	0.762	32	0.183 (0.126,0.261)	0.45	35	0.393 (0.256,0.558)	0.438
female	31	0.133 (0.074,0.154)		31	0.170 (0.129,0.375)		29	0.364 (0.232,0.483)	

4. Discussion

The high morbidity of T2DM and its various complications severely threaten human health and cause great burden to the social economy. In the advanced stage of T2DM, patients are often complicated with macrovascular, microvascular and neural complications, which severely weaken their life qualities. Multiple large-scale investigations have revealed that, intensive glucose-lowing therapy at the early phases of T2DM can benefit patients commendably by reducing incidences of macrovascular and microvascular complications[24, 25]. However, most patients at the early of T2DM stage are asymptomatic, they rarely go to hospital for the diagnosis and therapy. Meanwhile, current diagnostic methods show various deficiencies in the early diagnosis of T2DM:

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OGTT is the gold standard for diagnosis of T2DM, however, since the procedure is time-consuming and complicated, it's only considered when the
patient is highly suspected of T2DM; FPG is convenient, requiring only one measurement of plasma glucose, but the rate of missed diagnosis is very high [26]; hemoglobin A1c (HbA1c) test has not been standardized in Chinese hospitals. Hence a convenient, yet highly specific and sensitive diagnostic method is urgently needed to facilitate the early diagnosis of T2DM.

Because of the convenient sampling, low cost and high sensitivity, hematological markers play an important role in the diagnosis of many diseases. CircRNAs are abundant in body fluids [13, 14]. Among their diverse functions, the relatively important one is to serve as "miRNA sponge" that competitively binds miRNAs to exert a post-transcriptional regulation [10]. LncRNAs can also interact with miRNAs to regulate gene translation, suggesting potential correlation among the three types of non-coding RNAs [27]. Some lncRNAs and miRNAs have been proved to be involved in the occurrence and development of T2DM, and they also can be used as biomarkers for T2DM diagnosis [28, 29]. CircRNAs are much more stable than linear RNAs. Meanwhile, circRNAs expression levels are 10 times higher than that of linear RNAs in some tissues, making circRNAs a better biomarker.

The findings of our study showed that there were significant differences between the expression levels of circRNAs in the peripheral blood of T2DM patients and healthy subjects. 5 circRNAs were selected for validation, and the results indicated that hsa_circ_0054633 showed the highest diagnostic value for pre-diabetes and T2DM. The further validation of hsa_circ_0054633 was conducted in a cohort with a larger sample size, and it remained to have reliable diagnostic value, suggesting that hsa_circ_0054633 indeed had the potential to be a diagnosis biomarker for pre-diabetes and T2DM in clinical practice.

The field of circRNAs is quite a new area, so we haven't found any definite evidence to demonstrate the functions of hsa_circ_0054633. The results of gene ontology (GO) analysis showed that hsa_circ_0054633 not only participated in the biological processes such as cell cycle and mitotic cell cycle arrest, but also manifested strong correlation with catabolism of molecules. Cell cycle is the basic process of cellular life activities. The proliferation of β cells is regulated by cell cycle progress, and decreasing of β cell proliferation is the major cause of insufficient insulin secretion[30], which is also the basic of T2DM. Meanwhile, T2DM is a chronic metabolic disease characterized by disordered metabolism of carbohydrates, lipids and proteins. Therefore, it is speculated by our research team that hsa_circ_0054633 might participate in the pathogenesis of T2DM mainly by influencing cellular metabolism and cell cycle.

As far as we know, this study is the first to investigate the features of the expression profiles of circRNAs in peripheral blood of patients with T2DM, and to further validate the feasibility of hsa_circ_0054633 as a diagnostic biomarker for pre-diabetes and T2DM. The biomarker identified in this study (hsa_circ_0054633) can be easily tested using peripheral blood. The relatively low cost, high specificity and sensitivity making it a great aid to the early diagnosis of T2DM and pre-diabetes.

In our present research phase, only hsa_circ_0054633 was validated, while expression features of other circRNAs in T2DM and pre-diabetes patients remain to be explored. Also, this study was a single center study, with high geographic concentration of the subjects. Therefore, whether the populations in other regions have the similar circRNAs expression features are still to be confirmed. The results of our study require further verification in larger and more diverse cohorts.

5. Conclusions

In conclusion, this study is the first to prove that there are circRNAs expression differences in the peripheral blood between patients with T2DM and healthy subjects, and confirmed hsa_circ_0054633 as a potential biomarker for pre-diabetes and T2DM.

Acknowledgment: This work is supported by Department of Science and Technology of Henan province (grant number: 122102310620). We extend our sincere gratitude to all volunteers in our research, and the professors and clinicians to communicate with the patients.

Author contribution: Muwei Li conceived this study, Muwei Li, Peiyuan Hao, Xuejie Li and Zhenzhou Zhao collected the clinical samples, Xuejie Li and Zhenzhou Zhao designed and completed the experiment, collected and analyzed data, and drafted the manuscript. Chuanyu Gao, Muwei Li, Lixin Rao and Dongdong Jian revised the draft and finalized the manuscript. All authors have read and agreed on the final manuscript.

Author details: [1]Department of Cardiology, People's Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou, China. [2]Department of Cardiology, The First Affiliated Hospital of Zhejiang University, Zhejiang University, Hangzhou, China.

Conflicts of Interest: The authors declare no conflict of interest.

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