• Clinical Research •

TCERG1L hypermethylation is a risk factor of diabetic retinopathy in Chinese children with type 1 diabetes

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Abstract

● **AIM:** To identify the differential methylation sites (DMS) and their according genes associated with diabetic retinopathy (DR) development in type 1 diabetes (T1DM) children.

● **METHODS:** This study consists of two surveys. A total of 40 T1DM children was included in the first survey. Because no participant has DR, retina thinning was used as a surrogate indicator for DR. The lowest 25% participants with the thinnest macular retinal thickness were included into the case group, and the others were controls. The DNA methylation status was assessed by the Illumina methylation 850K array BeadChip assay, and compared between the case and control groups. Four DMS with a potential role in diabetes were identified. The second survey included 27 T1DM children, among which four had DR. The methylation patterns of the four DMS identified by 850K were compared between participants with and without DR by pyrosequencing.

● **RESULTS:** In the first survey, the 850K array revealed 751 sites significantly and differentially methylated in the case group comparing with the controls ($|\Delta\beta|>0.1$ and Adj.$P<0.05$), and 328 of these were identified with a significance of Adj.$P<0.01$. Among these, 319 CpG sites were hypermethylated and 432 were hypomethylated in the case group relative to the controls. Pyrosequencing revealed that the transcription elongation regulator 1 like (*TCERG1L*, cg07684215) gene was hypermethylated in the four T1DM children with DR ($P=0.018$), which was consistent with the result from the first survey. The methylation status of the other three DMS (cg26389052, cg25192647, and cg05413694) showed no difference (all $P>0.05$) between participants with and without DR.

● **CONCLUSION:** The hypermethylation of the *TCERG1L* gene is a risk factor for DR development in Chinese children with T1DM.

● **KEYWORDS:** DNA methylation; 850K array; pyrosequencing; diabetic retinopathy; type 1 diabetes; children
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INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a complex disease resulting from the interplay of genetic, epigenetic, and environmental factors[1-2]. Diabetic retinopathy (DR) is the most common and severe ocular complication of diabetes, and its pathogenesis has been studied extensively[3-4]. DNA methylation, along with histone acetylation and micro ribonucleic acid dysregulation, is an important epigenetic phenomena that results in altered gene expression[5]. Former researches indicated that DR is related with the DNA methylation changes of several genes[6-11]. However, most of these former researches focused on type 2 diabetes patients,

only two studies have reported a connection between DR and DNA methylation changes in T1DM adults[7-8]. So far, no such study has been published in T1DM children. The prevalence of DR in T1DM children is relatively lower than that in adults, which may result from a shorter disease duration. However, studying DR related DNA methylation in T1DM children is essential, because it may provide reference for preventing DR development in an early stage, even before its onset.

Several former studies indicated that T1DM children without DR have a significantly thinner macular retinal thickness compared with their healthy contemporaries[12-15], which is consistent with our previous findings in our study population. Moreover, we also found that the macular retinal thickness of T1DM children without DR decreased during a one-year follow-up (data not published yet). Retina thinning before the occurrence of microvascular changes might be resulted from diabetic retinal neurodegeneration[16]. Based on these evidences, we hypothesized that macular retina thinning could be used as a biomarker for early retinal damage related with T1DM before the onset of DR. Thus, in the present study, we first used the 850K BeadChip array to analyze the DNA methylation status of 40 T1DM children without DR, and screened for differential methylation sites (DMS) in the lowest 25% children with the thinnest macular retinal thickness comparing with the others. Four DMS with a potential role in diabetes were identified by the 850K BeadChip. Afterwards, we used the pyrosequencing assay to further investigate that four DMS in another study population of 27 T1DM children, including four with DR as case group, and the rest 23 without DR as controls. Thus, the DMS and their according genes related to the development of DR in T1DM children were obtained.

The aim of the present study was to identify the DMS and their according genes associated with DR development in T1DM children. This study may help to reveal novel DNA methylation markers, and to improve the current understanding, prevention and treatment of DR in T1DM patients.

SUBJECTS AND METHODS

Ethical Approval This study was a part of the Shanghai Children and Adolescent DM Eye study (clinical trial registration number: NCT 03587948)[17], and it included two hospital-based surveys (conducted in 2019 and in 2021 separately). This study was conducted in accordance with the tenets of the Declaration of Helsinki, and was approved by the ethics committees of the Shanghai General Hospital affiliated to Shanghai Jiao Tong University School of Medicine and Children's Hospital of Fudan University in Shanghai (approval number: 2016KY005 and LS No.01-2018, respectively). All of the participants and their guardians were fully informed about the procedures, and their informed consents were collected.

Setting and Participants The inclusion criteria were 1) age between 5y and 18y; 2) diagnosed with T1DM based on the World Health Organization diagnostic criteria[18]; 3) best corrected visual acuity no worse than 0.1 (logMAR visual acuity); 4) no previous eye surgery. Children with systemic diseases other than T1DM, and with severe eye diseases other than DR were excluded. Our research team consisted of three ophthalmologist, three optometrists, and 15 auxiliary staff.

Systemic and Ophthalmologic Exams Detailed examination procedures were described previously[17]. Briefly, participants' age, gender, diabetes duration, medication history, family history of diabetes, weight and height were collected though questionnaire. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Blood pressure was measured at the site. Routine blood tests results of the participants within 6mo at the hospital were collected. Total cholesterol and triglycerides were measured using an enzymatic assay. Hemoglobin A1C (HbA1c) was measured by ion exchange chromatography.

Comprehensive eye examinations were conducted in each of the participant in the ophthalmology clinic at Children's Hospital of Fudan University. Patients' eyelid, conjunctiva, cornea, iris, and lens were examined with a slit-lamp microscope (SL130; Zeiss, Germany), and the vitreous and fundus were examined with 90 D noncontact lens (90 D, Ocular, Bellevue, WA, USA) after cycloplegia. DR was diagnosed if microaneurysms, intraretinal hemorrhages, definite venous beading or prominent intraretinal microvascular abnormalities were observed[19]. Visual acuity was measured using the international standard logMAR visual acuity chart. The intraocular pressure was measured by a non-contact tonometer (model NT-530P, Nidek, Fremont, California, USA) before cycloplegia. The axial length (AL) was measured with IOL Master 700 (Carl Zeiss Meditec, Dublin, CA, USA). Cycloplegic refraction was performed using computer automatic optometry (KR-8900; Topcon, Tokyo, Japan) 20min after the cyclopentolate drops was used (1%; Alcon, Fort Worth, TX, USA). Swept source-optical coherence tomography (model DRI OCT Triton, Topcon, Tokyo, Japan) with a resolution of 1024 A scan/6 mm was used to measure the thickness of retinal layers with the center of macular. A radial scan with 12 lines centered on the fovea and separated by 30° was used to capture images. Built-in software was used to segment the layers and construct topographic maps. The average thickness of the retinal layers within 6-mm-diameter circle centered in the fovea was calculated automatically.

DNA Isolations and Bisulfite Treatment Genomic DNA was extracted from peripheral blood samples using DNeasy Blood & Tissue Kit (Qiagen 69506, Germany) according to its protocol. Afterwards, DNA was quantified by a Qubit 3.0

Fluorometer (ThermoFisher) and stored at -20°C. DNA 500 ng of each sample was used to bisulfite converted using EZ DNA Methylation Kits (Zymo Research, USA).

Infinium Human MethylationEPIC 850K BeadChip Assay (Illumina 850K) The Infinium MethylationEPIC 850K BeadChip assay (Illumina) was performed in accordance with Illumina's standard protocol. Bisulfite-converted DNA was amplified followed by enzymatic end-point fragmentation, precipitation and resuspension. Sample labeling, hybridization to chips and image scanning were performed and all 40 samples were processed on the same chipset to avoid a batch effect. No less than 10 biological repeats were conducted in each group.

Methylation Validation by Pyrosequencing PCR amplification of bisulfite-treated DNA was performed with primers designed using PyroMark Assay Design Software 2.0. Pyrosequencing was performed in accordance with the manufacturer's protocol on a PyroMark Q96 ID System (Qiagen). The Pyro Q-CPG software of the pyrosequencing device was used to automatically analyze the methylation status of each site. The sequences of the primers used for the four genes are listed in Table 1.

Data Analysis Statistical analyses were performed using R software (version 2.1.1). β value was used to represent DNA methylation level. The CpG sites with $|\Delta\beta|>0.1$ and *P*-value <0.05 in the case and control groups were considered significant. Student's *t*-test was used to compare differences in methylation levels and other parameters between the case and control groups. Chi-square test was used to compare categorical variables. Data were presented as mean±standard deviation for continuous variables, and as rates (proportions) for the categorical data. only the left eye parameters were used for statistical purpose. Raw *P*-value was adjusted using Benjamini method (R 4.0.0 *P*. adjust function with method='fdr') to control false discovery rate. $P<0.05$ was considered statistical significance. Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed using the scripts in Python to clarify the function and biological pathways of discovered DMS ($P<0.05$).

RESULTS

Characteristics of Participants The first survey was conducted in 2019, and a total of 40 T1DM children was included. Since none of these participants had DR, we set the lowest 25% participants (10/40) with the thinnest mean macular retinal thickness as the case group, and the rest as the control group. Table 2 listed the clinical characteristics of the participants from this first survey. There was no difference in age, gender, T1DM duration, BMI, HbA1c, triglycerides, total cholesterol, AL and spherical equivalent refraction (SER) between the case and control groups. The Infinium Human MethylationEPIC 850K BeadChip assay was used to analyze and to compare the genomic DNA methylation patterns between the case and control groups of this survey.

Table 1 Pyrosequencing primer sequences used in this study

CpG	Sequece 5′-3′
cg26389052	ACGTAGAGTT ATTATTAAAA ATTGAGT
cg07684215	TCGATTTAGA GTTACGTGTT AGGAGTTTTT TTTTTAGTTT
cg25192647	TTCGAGTAGT GTTTGGTAAT GGTAAGTA
cg05413694	TCGTTGGTCG TTGGGAGTAA TGTTTT

The second survey was conducted in 2021. A total of 27 T1DM children was included, and four of them had DR. For these four participants with DR, only retinal microaneurysms were observed. Thus, we set these four DR patients as the case group, and the rest as the control group. The clinical characteristics of these 27 participants is listed in Table 2, and there was no difference in age, gender, T1DM duration, BMI, HbA1c, triglycerides, total cholesterol, AL and SER between the case and control groups. For this second survey, pyrosequencing assay was used to further investigate the results of the 850K BeadChip array from the first survey.

Quality Control of the 850K BeadChip Array Data Genome-wide DNA methylation profiles of the 40 participants from the first survey were generated using the Illumina Human Methylation EPIC 850 BeadChip. The density distribution and the boxplots of the β-values were shown in Figure 1, which suggested that the overall distribution and concentration trend of the data in case group and control group were in a homogeneous distribution.

Comparison of the Genomic DNA Methylation Patterns Between Case and Control Groups Using the 850K BeadChip Array The 850K BeadChip analysis revealed that 751 sites were significantly and differentially methylated in the case group when compared with the controls ($|\Delta\beta|>0.1$ and Adj.$P<0.05$), while 328 of these were identified with a significance of Adj.$P<0.01$. Among these, 319 CpG sites were hypermethylated and 432 were hypomethylated in the case group relative to the control group. A volcano plot and a heat map were used to present the differentially methylated CpG sites between the case group and the control group (Figure 2A, 2B). The scatter plot of the average DNA methylation levels in the case group compared with those in the control group was presented in Figure 2C, with 432 hypomethylated sites and 319 hypermethylated sites shown in red. And the chromosomal distribution of the DMS were presented in Figure 2D, with the red denotes hypermethylated sites, and the blue denotes hypomethylated sites.

GO functional enrichment and KEGG signaling pathway analysis were used to further investigate the signaling path-

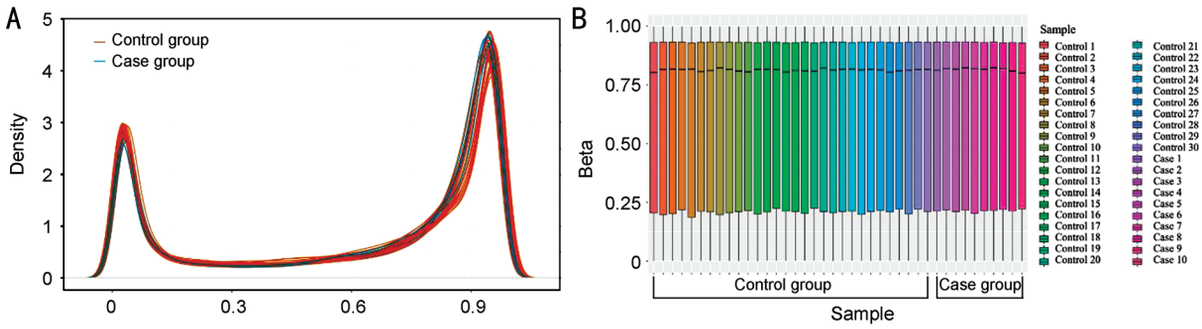

Figure 1 The distribution and concentration trend of the DNA methylation status A: The density distribution of the β-values in the case and control groups; B: The boxplots of the β-values distribution of the participants.

Table 2 The demographic systematic and ophthalmologic characteristics of the participated children from the first and the second surveys

Parameters	Survey 1			Survey 2		
	Control (*n*=30)	Case (*n*=10)	*P*	Control (*n*=23)	Case (*n*=4)	*P*
Age (y)	11.37±3.44	11.10±3.11	0.83	13.43±3.04	11.25±4.27	0.22
Male, *n* (%)	14 (46.67)	7 (70.00)	0.21	8 (34.78)	3 (75.00)	0.14
T1DM duration (y)	4.00±2.92	5.70±4.08	0.16	6.66±3.05	6.18±3.37	0.78
BMI	18.20±2.78	16.90±2.27	0.19	21.10±2.89	18.61±2.42	0.12
HbA1c	7.68±2.56	7.37±2.36	0.75	7.66±1.06	7.63±1.26	0.96
Triglycerides (mmol/L)	0.97±0.56	1.96±2.57	0.39	0.97±0.80	1.09±0.47	0.78
Total cholesterol (mmol/L)	4.62±1.08	4.96±1.80	0.53	4.19±0.75	4.65±1.33	0.32
AL (mm)	23.92±1.35	24.80±1.47	0.09	24.45±1.21	24.95±1.51	0.48
SER (diopter)	-1.20±2.72	-2.90±3.31	0.12	-2.91±2.65	-3.72±3.39	0.59
Macular retinal thickness (μm)	280±10	263±4	<0.01	278±13	270±14	0.30

T1DM: Type 1 diabetes mellitus; BMI: Body mass index; HbA1c: Hemoglobin A1c; AL: Axial length; SER: Spherical equivalent refraction.

ways associated with the DMS between the case and control groups to interrogate their biological functions. According to the criteria of $P<0.05$ and false discovery rate <0.05, the top 30 most significant major signaling pathways were identified (Figure 3A, 3B).

Validating the Results of the 850K BeadChip Array by the Pyrosequencing Assay We selected the Pyridoxal Kinase (*PDXK*, cg26389052), the Parkin RBR E3 Ubiquitin Protein Ligase (*PRKN*, cg25192647), the peroxisome proliferator-activated receptor-gamma (*PPARG*, cg05413694) and the transcription elongation regulator 1 like (*TCERG1L*, cg07684215) genes to further investigate the methylation status by pyrosequencing. Our selection was based on two reasons: first, the 850K array indicated that these four genes were significantly and differentially methylated between the case and control groups of the first survey, suggesting that they are associated with macular retina thinning, which is assumed as an early sign of T1DM related retinal damage before the onset of DR; second, former studies indicated that these four genes has a potential role in diabetes[20-22]. The most significant site is selected if multiple CpG sites are mapped to the same gene. Pyrosequencing revealed that only the *TCERG1L* (cg07684215) gene (P=0.018) was hypermethylated in the four T1DM children with DR, which was consistent with the result from the BeadChip analysis. The *PDXK* (cg26389052), *PRKN* (cg25192647) and *PPARG* (cg05413694) genes showed no difference (all $P>0.05$) in the methylation status between the case and control groups of the second survey (Figure 4).

DISCUSSION

In this study, we first used the 850K BeadChip array to screen for DMS related with retina thinning, which we hypothesized as a biomarker for early retinal damage before the onset of DR in T1DM children. Then, the pyrosequencing assay was used to further investigate whether or not the DMS identified by 850K BeadChip were associated with DR development in T1DM children. And our results indicated that the *TCERG1L* gene (cg07684215) is more hypermethylated both in the non-DR T1DM children with retinal thinning, and in the T1DM children with DR compared, with the controls.

So far, only two former studies have discussed DNA methylation changes associated with DR development in T1DM patients. Agardh *et al*[8] conducted a genome-wide analysis study in 58 T1DM adults, and identified differential DNA methylation of 349 CpG sites (representing 233 genes) in cases with proliferative DR compared with controls with no/mild DR. Chen *et al*[7] discovered 153 hypomethylated loci and 225 hypermethylated loci in cases with progressed retinopathy or macroalbuminuria comparing to controls without

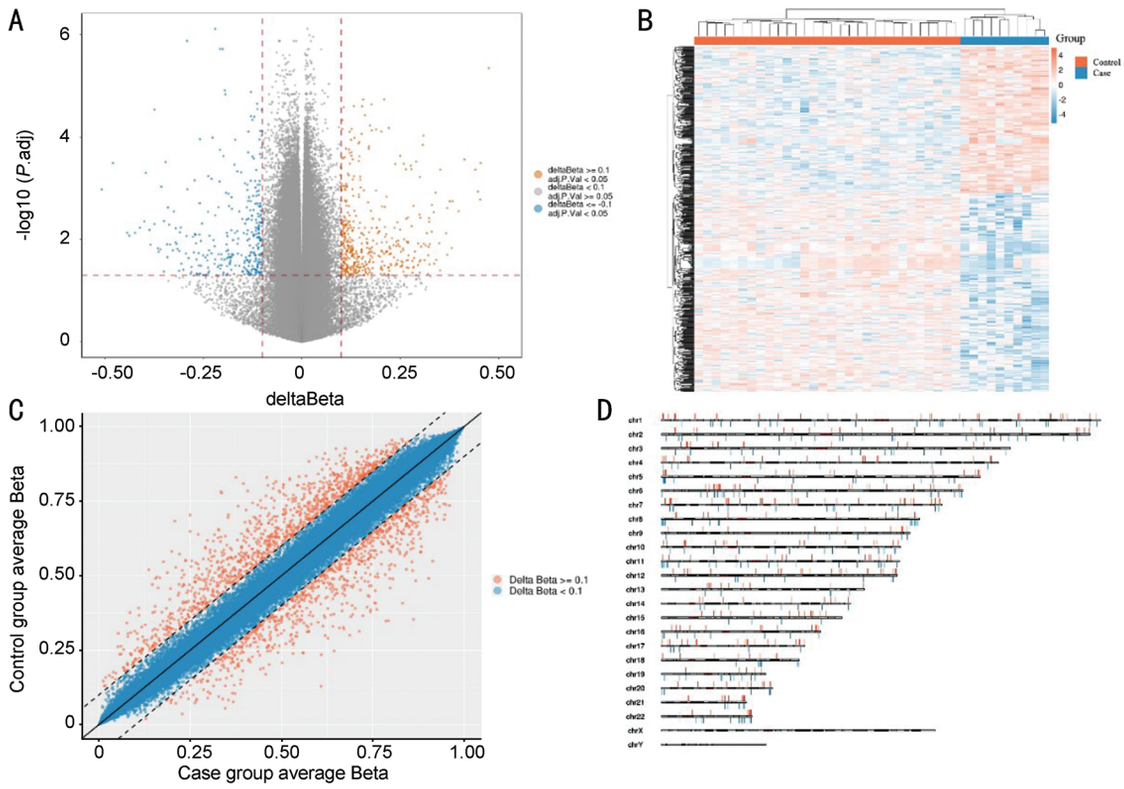

Figure 2 The DNA methylation pattern of the case group compared with the control group A: The volcano plot showing the hypomethylated genes (blue) and the hypermethylated genes (red) differentially methylated between the case and the control groups; B: Heat map showing the differentially methylated CpG sites (blue, CpGs with the lowest methylation levels; red, CpGs with the highest methylation levels) between the cases and the controls; C: Scatter plot showing the average DNA methylation level in the case group compared with those in the control group. The 319 hypermethylated sites and 432 hypomethylated are shown in red. D: The chromosomal distribution of differently methylated sites between the case and control groups (red denotes hypermethylated sites; blue denotes hypomethylated sites).

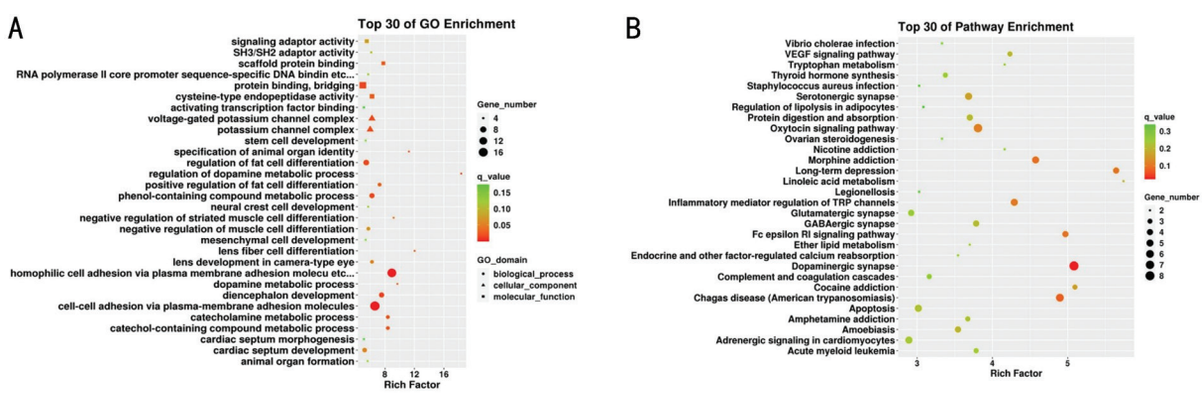

Figure 3 Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis results A: The top 30 functions from GO functional enrichment analysis; B: The top 30 pathways from KEGG pathway analysis.

complication progression in 63 T1DM adults using Infinium Human Methylation 450 Bead Chip Arrays. The study design and study population of these two studies are different from our research, and the *TCERG1L* gene was not identified by these two studies.

cg07684215 is located within the body of the *TCERG1L* gene at chr10:131092391-131311721 (hg38). As a paralog of *TCERG1*, the *TCERG1L* gene is a transcription elongation regulator that has been described to be involved in the pathogenesis of cancer and non-cancer-related diseases, including inflammatory bowel disease and colon cancer, suggesting that TCERG1L influences immunological pathways[23-25]. Moreover, previous genome-wide association studies have found that *TCERG1L* is associated with insulin resistance and type 2 diabetes in African Americans, West Africans and individuals of Indian ancestry[26-27]. Although T1DM belongs to chronic autoimmune disorders, as T-lymphocytes are activated to attack pancreatic β-cells[28], *TCERG1L* has not been reported previously to be associated with T1DM or DR.

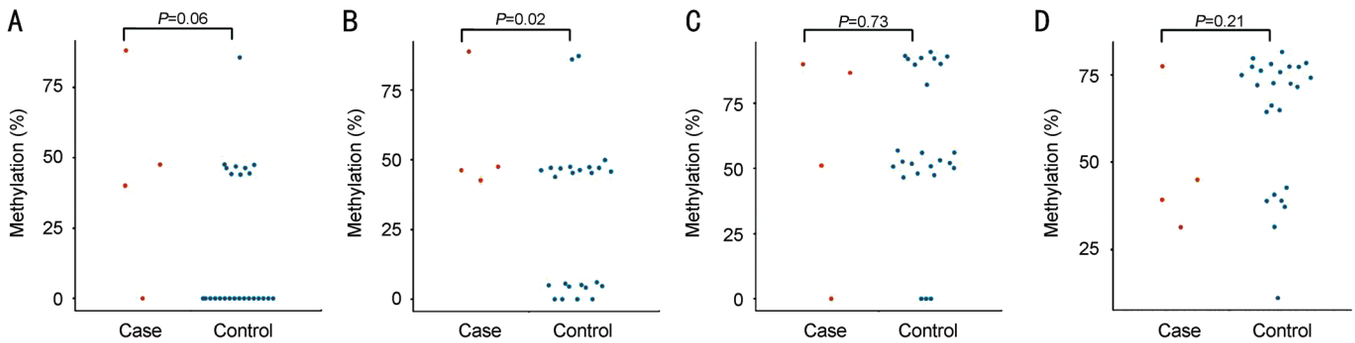

Figure 4 Using direct bisulfite pyrosequencing to investigate the DNA methylation patterns of four genes previously identified by the 850K BeadChip Type 1 diabetic children with retinopathy were set as case group, and those without retinopathy were set as control. A: Methylation level of cg26389052 (pyridoxal kinase, *PDXK*); B: Methylation level of cg07684215 (transcription elongation regulator 1 like, *TCERG1L*); C: Methylation level of cg25192647 (parkin RBR E3 ubiquitin protein ligase, *PRKN*); D: Methylation level of cg05413694 (peroxisome proliferator-activated receptor-gamma, *PPARG*).

We did not find any association between DR and the methylation status of the *PDXK*, *PRKN*, and *PPARG* genes by the pyrosequencing assay. This inconsistency between the results of the 850K BeadChip array and the pyrosequencing assay is not unpredicted. The most probable reason of the inconsistency is the different grouping methods we used in the two surveys. As explained earlier, because none of the participants had DR in the first survey, we set the lowest 25% participants (10/40) with the thinnest mean macular retina thickness as the case group. This grouping method is based on the hypothesis that macular retina thinning could be used as a biomarker for T1DM related early retinal damage before the onset of DR. Although the hypothesis is supported by some former published researches[12-15] and our previous findings, macular retina thinning certainly does not equal to DR development. Other possible reasons for this inconsistency are mainly caused by the limitation of genome-wide DNA methylation tests, because DNA methylation variability can be composed of both biological and technical sources[29].

This study has several limitations. First, the number of the participants was small. Although we included totally 67 participants in the two surveys, which is comparable with the former T1DM studies[7-8], only 4 of them had DR. Because of the shorter disease duration, the prevalence of DR in T1DM children is significantly lower than that in the adults[30]. Small case group may affect the result. We will repeat and improve the study once more T1DM children with DR appear. Second, conclusions of this study may only apply to Chinese children with T1DM. Future studies analyzing the association between *TCERG1L* and DR in T1DM patients with a larger case group and a different study population may reveal further evidences.

In conclusion, our results reveals that the hypermethylation of the *TCERG1L* gene is a risk factor for DR development in Chinese children with T1DM. This study is the first to report the relation between DR and DNA methylation changes in T1DM children, and may provide reference for the prevention, screening and early treatment of DR in T1DM patients.

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