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Circular RNA expression profiles in placental villi from women with gestational diabetes mellitus

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ABSTRACT

Circular RNAs (circRNAs) have recently been shown to exert their effects on multiple pathological processes by acting as microRNA (miRNA) sponges. However, the roles of circRNAs in gestational diabetes mellitus (GDM) are largely unknown. This study aimed to identify the circRNAs involved in GDM and predict their potential biological functions. We first performed next-generation sequencing (NGS) to generate unbiased placental villi circRNA expression profiles of GDM and normal controls. In total, 48,270 circRNAs from the placental villi of the two groups were sequenced. Of these, 227 circRNAs were significantly up-regulated and 255 circRNAs were significantly down-regulated. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway analyses demonstrated that glycometabolism and lipometabolism processes, which are important in GDM development, were significantly enriched. Further analysis showed that most of the circRNAs harbored miRNA binding sites, and some were associated with GDM. These results showed that circRNAs are aberrantly expressed in the placental villi of GDM patients and play potential roles in the development of GDM.

1. Introduction

Gestational diabetes mellitus (GDM) is characterized by any degree of glucose intolerance during pregnancy. The prevalence of GDM varies between countries and even between regions within a country [1,2], and GDM affects approximately 3.0–21.2% of the pregnancies in Asian countries [2]. Recent studies reported that women with GDM, the risks of stillbirth, fetal macrosomia and dystocia birth canal injury are increased and the rate of cesarean section is up to 23.7% [3]. Moreover, postpartum women with GDM present higher catalase levels than do controls, and these increases are positively correlated with glucose intolerance [4]. For the offspring of GDM mothers, the adverse maternal environment may increase the risk of metabolic diseases, including type-2 diabetes, obesity, and cardiovascular disease, later in life [5,6].

Circular RNA (circRNA) is a novel type of non-coding RNA (ncRNA) that forms a unique circular covalently closed structure [7]. Some specific circRNAs can function as a miRNA sponge to regulate the transcription of miRNA-targeted genes [8], and have close clinical and pathological correlations with human diseases, such as tumors, neurodegenerative disorder and cardiovascular disease [9–11]. For example, Ubiquitin-conjugating enzyme E2A (UBE2A) coordinates the clearance of amyloid peptides in Alzheimer's disease (AD), UBE2A deficiency is linked to deficits in circRNA for miRNA-7 (ciRS-7)-targeted “sponging events” [10]. Qian et al. [12] explored circRNA expression profiles in human placental tissues from patients with preeclampsia and proposed that circRNAs may contribute to preeclampsia pathogenesis. Therefore, we speculate that a similar circRNA phenomenon was also apparent in GDM.

In this study, we explored the circRNA expression profiles of placental villi from GDM women and predicted the potential functions that circRNAs exerted in the development of GDM. Our work provides novel insight into the pathological mechanisms of GDM.

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2. Materials and methods

2.1. Ethics statement

This study was approved and supervised by the Ethics Committee of Nanjing Maternal and Child Health Hospital (Nanjing, China), and the ethical permit number is [2015]11. All the experiments were performed in accordance with the Code of Ethics of the World Medical Association. Written informed consents were obtained from all the volunteers for research purposes.

2.2. Clinical specimens

The placental villi for this study were collected from 60 maternal volunteers (30 GDM cases and 30 normal controls) who underwent low cesarean section in Nanjing Maternal and Child Health Hospital from May 2016 to June 2017. All the volunteers had full-term infants (gestational age ranged from 38 to 41 weeks), and the average maternal age was 29.1 years. The clinical data for women with and without GDM are presented in Table 1.

The fresh villi were obtained from the maternal surfaces of the placentas and snap-frozen in liquid nitrogen immediately before several quick and gentle washes with PBS. These specimens were subsequently stored in liquid nitrogen until use.

2.3. Circular RNA next-generation sequencing (NGS)

Six placental villi, including 3 GDM and 3 normal samples, were selected for NGS analysis of circRNAs by Biotechnology (Shanghai, China). Briefly, after total RNA extraction and ribosomal RNA removal, a cDNA library was constructed in line with the TruSeq library preparation protocol, and sequencing was performed on an Illumina HiSeq 2500 V4 (Applied Biosystems, USA) platform for 100 cycles with a paired-end program. Sequence reads mapping against the reference genome GRCh38/hg38 with UCSC gene annotation was performed with BWA-MEM. The back-spliced junction reads for circular RNAs were obtained using CIRI software [13].

2.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted using QIAzol Lysis Reagent (Qiagen, USA) and an RNeasy Mini Kit (Qiagen,USA) according to the manufacturer's instructions. RNA quantity and quality were measured with a Nano Drop ND-1000 spectrophotometer (Thermo, USA). RNA integrity was also assessed by standard denaturing 1% agarose gel electrophoresis. Subsequently, the RNA was digested using RNase R (Epicenter Biotechnologies, USA) and purified. cDNA was synthesized according to the manufacturer's protocol of PrimeScript RT reagent with gDNA Eraser (TaKaRa, Japan).

Table 1
Clinical data for the GDM patients and normal controls.

	GDM (n = 30)	NC (n = 30)	P-value
Maternal age (years)	29.20 ± 2.61	29.07 ± 2.85	>0.05
Median maternal BMI (kg/m^2)	25.90 ± 1.84	25.13 ± 1.70	>0.05
Gestational age (weeks)	39.20 ± 0.89	39.33 ± 0.92	>0.05
Birth weight (kg)	3.51 ± 0.34	3.29 ± 0.21	<0.05
Fasting plasma glucose/mmol/L	5.31 ± 0.51	4.38 ± 0.38	<0.01
1 h plasma glucose/mmol/L	10.92 ± 1.01	9.00 ± 0.54	<0.01
2 h plasma glucose/mmol/L	8.91 ± 0.73	6.93 ± 0.74	<0.01

The P-value refers to comparisons between the "GDM" and "NC" groups. The data are presented as the mean ± SD. BMI, body mass index; GDM, gestational diabetes mellitus; NC, normal control.

2.5. Divergent primer design and Regular-PCR (R-PCR)

The specific divergent primers were designed using Primer 5.0 to amplify the circular transcripts through head-to-tail splicing. All the primers were synthesized with Realgene (Nanjing, China). The primer sequences are listed in Supplementary Table S1.

R-PCR was performed based on the recommendations of Premix Taq (TaKaRa, Japan) in three different temperature gradients (56 °C, 59 °C, 62 °C) to determine the best annealing temperatures. The amplification products were visualized by gel electrophoresis with 3% ethidium bromide-stained agarose gels under the cirteira: only one bright band appeared with no primer dimers or non-specific amplification products and when the molecular weight was equal to the size of the target fragment.

2.6. Quantitative real-time PCR (qRT-PCR)

After determining the best annealing temperatures, qRT-PCR was performed to measure the relative circRNA expression levels using PowerUP SYBR Green Master Mix (Applied Biosystems, USA) on a Life Tech-ViiA7 system (Applied Biosystems, USA). Glyceraldehyde phosphate dehydrogenase (GAPDH) served as the internal control, the relative expression level of each circRNA was calculated with the $2^{-\triangle\triangle Ct}$ method.

2.7. GO and KEGG biological pathway analysis

The parental gene functions of the differentially expressed circRNAs were analyzed using DAVID Bioinformatics Resources 6.8 [14] (https://david.ncifcrf.gov/home.jsp). GO analysis of the parental genes was performed based on three terms, namely, biological processes (BP), cellular components (CC) and molecular functions (MF); the related biological pathways were analyzed by KEGG. We regarded the −log (P-value) as the enrichment score that indicated the significance of correlation.

2.8. Annotation for circRNA/miRNA interactions

The circRNA/miRNA interactions were predicted using miRanda [15] (http://www.microrna.org/microrna/home.do/). With the database, we searched miRNA response elements (MREs) on circRNAs, then selected miRNAs based on the seed-match sequences.

2.9. Statistical analyses

Statistical analyses were performed with SPSS 19.0 and GraphPad prism 5.0. All the data are displayed as the mean ± SD for triplicate independent measurements. Student's t-test was used to assess the differences between experimental groups. Differences with P-values < 0.05 were considered statistically significant.

3. Results

3.1. General characteristics of circRNAs

To study the general characteristics of all the circRNAs in human placenta villi, we performed a preliminary analysis of all the sequencing results. A total of 48,270 circRNAs were evaluated, including 14,686 circRNAs known in circBase (http://circrna.org/) and 33,584 circRNAs that were first identified in this study. Fig. 1A shows the reads distribution of all circRNAs. These circRNA parental genes were widely scattered on almost all human chromosomes (Fig. 1B). In our summary of the categories of 48,270 circRNAs, there

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