Cardiovascular Diabetology

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Predicting gestational diabetes mellitus risk at 11–13 weeks' gestation: the role of extrachromosomal circular DNA

Jin Wang[1†], Pengyu Huang[2†], Fei Hou[1], Dongdong Hao[3], Wushan Li[4] and Hua Jin[1*]

Abstract

Background Gestational diabetes mellitus (GDM) significantly impacts maternal and infant health both immediately and over the long term, yet effective early diagnostic biomarkers are currently lacking. Thus, it is essential to identify early diagnostic biomarkers for GDM risk screening. Extrachromosomal circular DNA (eccDNA), being more stable than linear DNA and involved in disease pathologies, is a viable biomarker candidate for diverse conditions. In this study, eccDNA biomarkers identified for early diagnosis and assessment of GDM risk were explored.

Methods Using Circle-seq, we identified plasma eccDNA profiles in five pregnant women who later developed GDM and five matched healthy controls at 11–13 weeks of gestation. These profiles were subsequently analyzed through bioinformatics and validated through outward PCR combined with Sanger sequencing. Furthermore, candidate eccDNA was validated by quantitative PCR (qPCR) in a larger cohort of 70 women who developed GDM and 70 normal glucose-tolerant (NGT) subjects. A ROC curve assessed the eccDNA's diagnostic potential for GDM.

Results 2217 eccDNAs were differentially detected between future GDM patients and controls, with 1289 increased and 928 decreased in abundance. KEGG analysis linked eccDNA genes mainly to GDM-related pathways such as Rap1, MAPK, and PI3K-Akt, and Insulin resistance, among others. Validation confirmed a significant decrease in eccDNA PRDM16^{circle} in the plasma of 70 women who developed GDM compared to 70 NGT women, consistent with the eccDNA-seq results. PRDM16^{circle} showed significant diagnostic value in 11–13 weeks of gestation (AUC = 0.941, $p < 0.001$).

Conclusions Our study first demonstrats that eccDNAs are aberrantly produced in women who develop GDM, including PRDM16^{circle}, which can predict GDM at an early stage of pregnancy, indicating its potential as a biomarker.

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Keywords Gestational diabetes mellitus, Extrachromosomal circular DNA, Predictive biomarker

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Introduction

Gestational diabetes mellitus (GDM) is a common pregnancy complication, defined as varying degrees of abnormal glucose tolerance first identified during pregnancy [1]. The incidence of GDM varies globally, ranging from 1.8 to 31%, and has significantly increased recently [1–3]. GDM affects both the woman and offspring during and after pregnancy [4, 5]. For women, GDM is linked to pre-eclampsia, polyhydramnios, cesarean section, postpartum hemorrhage, and postpartum infections. For the fetus, increased risks include neonatal hypoglycemia, hypocalcemia, fetal overgrowth, preterm delivery, and stillbirth. Furthermore, GDM poses long-term health risks for both mother and child [1, 4, 5]. It increases the likelihood of obesity, diabetes, metabolic syndrome, and other cardiometabolic issues, which can impact the health and life quality of subsequent generations.

Currently in China, GDM screening occurs between 24 and 28 weeks of gestation via a 75-g oral glucose tolerance test (OGTT), with diagnoses made according to International Association of Diabetes in Pregnancy Study Group (IADPSG) criteria [6]. However, the test, requiring three venous blood samples over two hours, often causes gastrointestinal reactions and increases the burden on women not at risk for GDM. Moreover, this test may underdiagnose women at high risk for GDM, potentially leading to missed diagnoses [7]. Clinical practice indicates that when GDM is diagnosed between 24 and 28 weeks of gestational, maternal and fetal health is already compromised to varying degrees prior to intervention, although symptoms can improve with active intervention [4, 5]. Therefore, predicting the risk of GDM at the early stage of pregnancy and advancing its diagnosis are crucial for reducing both its incidence and associated harms through early detection and intervention.

In predicting GDM, potential predictive biomarkers include glycosylated hemoglobin, triglycerides, and pregnancy-associated plasma protein A (PAPP-A), as well as molecular biomarkers such as microRNAs (miRNAs), exosomes, DNA methylation, and polymorphisms (SNPs) [1, 2, 8, 9]. Despite these biomarkers' potential, most studies have small sample sizes, and the biomarkers' reproducibility and reliability require further validation. Additionally, other studies have developed predictive models for GDM using electronic health records and laboratory tests [10–12]. However, these results are preliminary and demonstrate insufficient sensitivity and specificity. Currently, no biomarkers or predictive models for GDM are available that are clinically useful or widely accepted [8].

Extrachromosomal circular DNA (eccDNA) is derived from yet distinct from chromosomal DNA and contains tens to millions of base pairs organized in a circular form [13]. eccDNA is highly heterogeneous in length, amount, and origin, due to varying cell and tissue types and genetic backgrounds, and plays a key role in biological processes such as cancer development, reproduction, aging, and genomic diversity [13–16]. The closed circular structure of eccDNA provides greater stability, and increased sensitivity and specificity in comparison to the detection of linear DNA, making it a promising source of potential biomarkers [13, 17, 18]. Studies have shown that eccDNA in body fluids such as plasma, serum, and urine could serve as biomarkers for predicting, diagnosing, and monitoring diseases [18–20].

In this research, peripheral blood samples were obtained for prospective evaluations from pregnant women at 11–13 weeks' gestation. The profiles of eccDNA in women who would and would not later develop GDM were characterized, revealing the functional linkage of eccDNA. This analysis identified a novel eccDNA named PRDM16^{circle}, which was present at significantly lower levels in 70 women who developed GDM compared to 70 NGTs. These significant findings are expected to improve the early prediction and diagnosis of GDM.

Materials and methods

Sample collection

The study utilized a birth cohort from Jinan. Women with singleton pregnancies at 11^{+0} to 13^{+6} weeks of gestation were re-recruited at Jinan Maternity and Child Care Hospital, where their peripheral venous blood was collected at the same time. Regular antenatal examinations were performed for the recruited pregnant women. Pregnant women underwent a 75 g OGTT between 24 and 28 weeks of gestation. GDM diagnosis followed International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria: fasting glucose ≥ 5.11 mmol/L, 1-h glucose ≥ 10.00 mmol/L, or 2-h glucose ≥ 8.50 mmol/L [1]. Ethics approval was obtained from Jinan Maternity and Child Care Hospital's review committee, in line with the Declaration of Helsinki (No. IRB KY-23-57). *Trial registration* ChiCTR2300075971, http://www.chictr.org.cn. Registered 20 September 2023. All participants provided signed informed consent. Women with fetal growth restriction, hypertensive disorders, or pre-gestational diabetes were excluded from the study. Control participants, without complications, were matched 1:1 with cases based on gestational age during blood collection. Finally, 150 women with single pregnancy—75 who developed GDM (GDM group) and 75 with normal glucose tolerance (NGT group)—were collected in this study. Hospital records provided clinical data on maternal and newborn outcomes (Table 1).

Table 1 Characteristies of participants in the study

Variables	Sequencing samples			Validation samples		
	GDM ($n=5$)	Control ($n=5$)	*p*-value	GDM ($n=70$)	Control ($n=70$)	*p*-value
Maternal age (y)	35.2 ± 2.8	29.8 ± 1.6	0.006	34.0 (29.0, 36.0)	29.5 (27.0, 32.3)	< 0.001
Gestational age at sampling (weeks)	12.5 ± 0.5	12.5 ± 0.7	0.873	12.4 (12.1, 13.2)	12.3 (11.9, 13.0)	0.406
Prepregnancy BMI (kg/m^2)	23.8 ± 0.9	21.2 ± 1.5	0.011	23.4 (22.4, 24.8)	22.2 (20.4, 24.0)	< 0.001
HbA1c (%)	5.7 ± 0.1	5.2 ± 0.2	0.003	5.6 (5.3, 6.0)	5.3 (5.1, 5.5)	< 0.001
Fasting plasma glucose (mmol/L)	5.2 ± 0.2	4.5 ± 0.2	0.001	4.6 (4.4, 4.8)	4.6 (4.4, 4.7)	0.215
Insulin (pmol/L)	13.6 ± 7.0	5.9 ± 1.3	0.067	10.3 ± 2.9	8.6 ± 2.4	< 0.001
HOMA-IR	3.2 ± 1.7	1.2 ± 0.3	0.054	2.1 ± 0.6	1.7 ± 0.5	< 0.001
OGTT-fasting (mmol/L)	5.6 ± 0.4	4.6 ± 0.4	0.002	5.2 (4.7, 5.5)	4.7 (4.5, 4.9)	< 0.001
OGTT-1 h (mmol/L)	10.1 ± 1.5	6.7 ± 1.4	0.006	10.0 (9.3, 10.8)	6.6 (6.0, 7.6)	< 0.001
OGTT-2 h (mmol/L)	9.5 ± 1.4	6.2 ± 0.6	0.001	8.7 (8.1, 9.2)	6.5 (5.6, 7.4)	< 0.001
Gestational age at delivery (weeks)	37.7 ± 1.6	39.7 ± 0.5	0.030	38.3 (37.4, 39.3)	39.1 (39.2, 39.6)	0.015
Birth weight (g)	3342.0 ± 679.0	3376.0 ± 310.0	0.921	3385.6 ± 612.2	3275.7 ± 433.8	0.223

All variables were investigated or measured at 11–13 weeks' gestation, except for OGTT-fasting, OGTT-1 h, and OGTT-2 h, which were measured between 24 and 28 weeks of gestation. Non-normally distributed continuous data were presented as medians with interquartile ranges, while normally distributed continuous data were expressed as means ± standard deviations (SD)

GDM, gestational diabetes mellitus; BMI, body mass index; HbA1c, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; OGTT, oral glucose tolerance test

Collection of peripheral blood samples

5 mL of peripheral venous EDTA blood samples were obtained during 11 to 13 weeks of gestation and then centrifuged at 1600×*g* for 10 min at 4 °C. The plasma was then aspirated into an EP tube, subsequently centrifuged at 16,000×*g* for 10 min at 4 °C, and later stored at − 80 °C.

Circle-Seq library preparation and sequencing

High-throughput eccDNA sequencing was performed by CloudSeq Biotech Inc. (Shanghai, China) based on the published procedures with minor modifications [21]. Circle-Seq was performed on samples from 5 GDM patients and 5 controls. In brief, plasma DNA extraction was performed with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) following the provided guidelines. Plasma DNA underwent a 5-min enzymatic digestion at 37 °C with Plasmid-Safe ATP-dependent DNase to eliminate linear DNA. This DNA was then purified and concentrated using the MinElute Reaction Cleanup Kit (Qiagen, Germany). The processed DNA was subsequently utilized for constructing libraries using the Nextera XT DNA Library Preparation Kit (Illumina, United States). The libraries were sequenced as 1×150-bp paired-end reads on a NovaSeq 6000 sequencer for high-throughput sequencing.

Sequencing analysis of eccDNA

Sequencing was conducted on an Illumina NovaSeq 6000, achieving a Q30 quality standard, which corresponds to a base call accuracy of 99.9%. Splices and low-quality reads were removed using Cutadapt software (v1.9.1), resulting in high-quality reads. These reads were then aligned to the human reference genome (HG19) using Bwa software (v0.7.12). Subsequently, Circle-Map software (v1.1.4) was employed for further analysis, clean reads were further analyzed to identify eccDNA in all samples. Samtools (v2.0) quantified softclip reads overlapping with breakpoints, using raw counts for softclip reads. Differentially abundant eccDNAs were identified based on fold change (> 2 or < 1/2) and a *p*-value (< 0.05) between groups. Bedtools (v2.27.1) analyzed identified and differentially abundant eccDNAs. Genetic annotation and analysis utilized Bedtools (v2.27.1), followed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of associated genes.

Validation of PRDM16^{circle} using outward PCR and Sanger sequencing

Experimental validation of PRDM16^{circular} DNA at Chr1:3092613–3092863 was conducted. Briefly, DNA from blood specimens with high abundant in Circle-Seq was extracted and subjected to digestion with Plasmid-Safe ATP-dependent DNase to eliminate linear DNA, as described before. The obtained DNA underwent purification and enrichment following the previously outlined methods. Primers facing outward were designed to target eccDNA junctions using NCBI and Primer Premier 5.0 (PREMIER Biosoft, USA), targeted eccDNA junctions (Additional file 1: Table S1). Each 50 µl PCR system contained 4 µl template, 10 µl primer, 25 µl PCR master mix (GenSeq Biotech, Inc.), 11 µl double-distilled water, and was run for 30 cycles. PCR conditions included: 98 °C for 30 s, followed by 30 cycles at 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min and then held at 4 °C. PCR products were evaluated via 2% agarose gel electrophoresis and purified using the QIAEX II gel extraction kit (Qiagen), and subjected to Sanger sequencing (Shanghai Sangon Biotech, China).

qPCR verification of PRDM16^{circle}

Quantitative real-time PCR (qPCR) was used to validate PRDM16^{circle} using outward-facing primers (additional file 1: Table S1) in the sequencing samples. Additionally, the presence of PRDM16^{circle} was validated in a new cohort, including 70 women who developed GDM and 70 NGT individuals. Briefly, the pGEX-5X-2 plasmid was added to plasma samples at a ratio of 1.2×10^6 copies/ml prior to eccDNA extraction and served as an internal control [22]. DNA extraction, purification, and enrichment followed the methods described above. The PCR reaction mixture totaled 10 µl, including 2 µl of DNA template, 0.5 µl of both forward and reverse primers (10 mM each), 5 µl of 2× SYBR Green master mix, and 2 µl of double-distilled water. The qRT-PCR reactions were carried out under standard PCR conditions. All experiments were performed in triplicate, and the average CT value from the three wells was normalized to the pGEX-5X-2 internal control (ΔCt = Ct average PRDM16^{circle}—Ct average pGEX-5X-2). Relative levels were determined using the $2^{-\Delta\Delta Ct}$ method.

Statistics analysis

All statistical analyses and visualizations in this study were conducted using R version 4.3.2, SPSS version 22.0 and GraphPad Prism version 8.0. Continuous data not following a normal distribution were presented as medians and interquartile ranges, while data conforming to normal distribution were shown as means ± SD. T-tests were applied to compare normally distributed data across groups, while the Mann–Whitney U test was used for data that did not follow normal distribution.

To evaluate the predictive impact of plasma eccDNA PRDM16^{circle} and other clinical parameters on the risk of developing GDM, a predictive model incorporating multiple variables was constructed. Firstly, variables significantly associated with GDM, such as gestational age at sampling, maternal age, pre-pregnancy body mass index (BMI), levels of glycated hemoglobin (HbA1c), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), insulin levels, and fasting plasma glucose, were identified through group difference analysis. These variables were further analyzed using univariate logistic regression to assess the strength of their associations with the incidence of GDM. Subsequently, the least absolute shrinkage and selection operator (LASSO) method was employed to optimize the selection of the most predictive variables. Based on the outcomes of the LASSO regression, four variables were chosen for the construction of a nomogram model. Scores were assigned to each predictor in the nomogram, which integrated these scores through Binary logistic regression to compute a total score for predicting the risk of GDM. Sensitivity analysis for these four variables was conducted using SALib, an open-source Python library for sensitivity analysis. Additionally, the diagnostic efficacy of the model was to be assessed by calculating the area under the receiver operating characteristic (ROC) curve (AUC). All statistical analyses were significant at a p-value threshold of 0.05.

Results

The plasma eccDNA biomarker discovery workflow and baseline characteristics of subjects

The experimental design for the current plasma eccDNA biomarker discovery and validation study is shown in Fig. 1. The characteristics of the women and their newborns are detailed in Table 1. None of the pregnant women had gestational hypertension, fetal growth restriction (FGR), thyroid disorders, liver or kidney disease, or severe infections. There were no significant variations in gestational age at sampling, fasting plasma glucose, or neonatal weight between the GDM and NGT groups (all $p>0.05$). Notably, significant differences were noted in maternal age, prepregnancy BMI, HbA1c levels, insulin levels, HOMA-IR, OGTT results, and gestational age at delivery between the two groups (all $p<0.05$).

The landscape of eccDNA

In this research, each specimen produced about 250 million Circle-seq reads, with quality control data provided in Additional file 2: Table S2. The genome-wide profile of eccDNAs was analyzed in plasma samples from women who would later develop GDM and those who would not, and the Circle-seq data are available in the Genome Sequence Archive (GSA, https://ngdc.cncb.ac.cn/gsa/) under accession number HRA007356. From the ten plasma samples, 31,630 eccDNAs were detected, indicating their prevalence in human plasma. The Venn diagram indicates that 11,164 eccDNAs were unique to GDM group, 15,740 unique to NGT group, and 4726 detected in both groups (Fig. 2A). Upon examining the frequency of eccDNAs per megabase on each chromosome, chromosomes 19, 17, and 20 exhibited higher eccDNA frequencies than others (Fig. 2B). EccDNA frequencies per megabase were generally lower in the GDM group compared to the NGT group, with the exception of chromosome 9. There was a notable correlation between the number of coding genes and eccDNAs ($r=1$, $p<0.001$; Fig. 2C), suggesting a role for coding gene features in eccDNA formation. Plasma eccDNA molecules were mapped to various genomic and repetitive elements (Fig. 2D, E). EccDNAs predominantly originated from the CpG and 5′UTR regions, and from repetitive elements like short interspersed elements (SINE) and long interspersed elements (LINE). The length distribution of eccDNAs showed similar length distributions between the GDM and NGT groups, concentrated between 100 and 420 bp (Fig. 2F). Plasma eccDNAs demonstrated

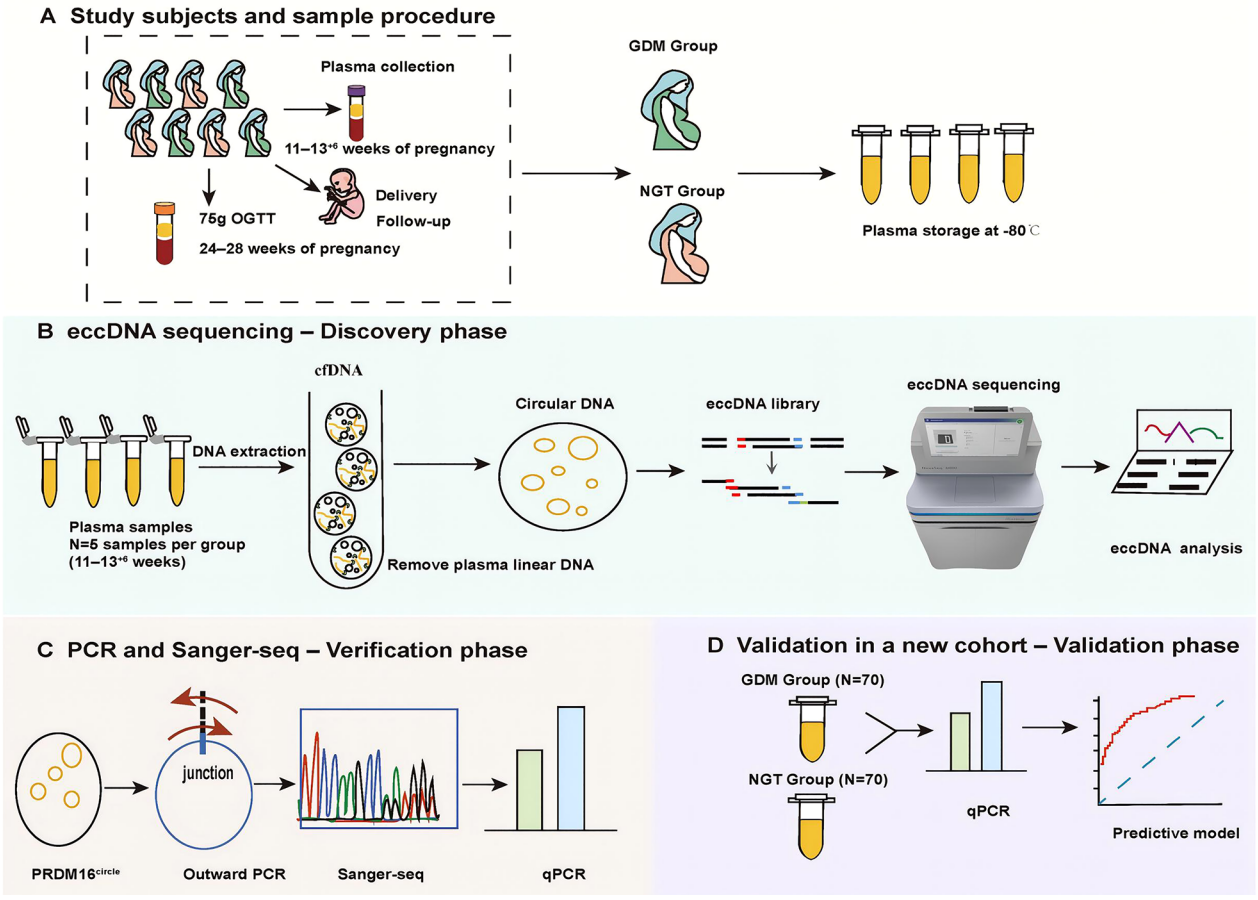

Fig. 1 The workflow for plasma eccDNA biomarker discovery for GDM. **A** Plasma samples collected from participants were stored at −80 °C for later analysis. **B** DNA, including both linear and circular forms, was extracted from plasma, followed by the digestion of linear DNA using exonuclease V and subsequent RCA of eccDNAs. The samples were then analyzed using Circle-Seq, offering detailed insights into the eccDNA profiles. **C** Verification involved outward PCR targeting specific eccDNA junctions, followed by Sanger sequencing to confirm sequence accuracy, and qPCR to quantify the presence of eccDNA. **D** The eccDNA biomarker was validated in 70 women who developed GDM and 70 NGT subjects. GDM, gestational diabetes mellitus; NGT, normal glucose tolerance; RCA, rolling circle amplification

two distinct peaks at 170 bp and 339 bp, with the 170 bp peak being more prominent. Cumulative frequency plots revealed a significant disparity in eccDNA lengths between the two groups ($p<0.001$) (Fig. 2G). A total of 2217 eccDNAs showed differential abundance between the GDM and NGT groups, with 1289 being more prevalent and 928 being less prevalent (fold change ≥ 2.0 or ≤ 0.5; $p<0.05$, Fig. 2H–J).

Biological function of eccDNAs

To identify the signaling pathways and functions impacted by GDM, analyses using the 'Clusterprofiler' R package were conducted on differentially abundant eccDNAs, focusing on Gene Ontology (GO) terms and KEGG pathways. Enrichment was noted in GO terms related to multicellular organism development, developmental processes, system development, and cell differentiation, etc. (Fig. 3A–F). 230 pathways associated with increased eccDNA genes were identified, including Protein digestion and absorption, Glycosphingolipid biosynthesis (ganglio series), Rap1 signaling, and cGMP-PKG signaling pathways, among others (Table 2). 213 pathways linked to decreased eccDNA genes were identified, including the Hippo, MAPK, AMPK signaling pathways, and Insulin resistance, among others (Table 2). These results suggest that these signaling pathways may be associated with the occurrence and development of GDM.

Plasma eccDNA PRDM16^{circle} level is decreased in women who developed GDM

Considering the impracticality of verifying all abnormally regulated eccDNAs, our study prioritized eccDNA PRDM16$^{circle\ chr1:3092613-3092863}$ (referred to as PRDM16^{circle}) for further validation of the eccDNA-seq results. PRDM16^{circle} was chosen for its significant fold change of 0.03, p-value of 0.007, gene locus at Chr1:3092613–3092863, and its possible role in GDM's molecular mechanisms.

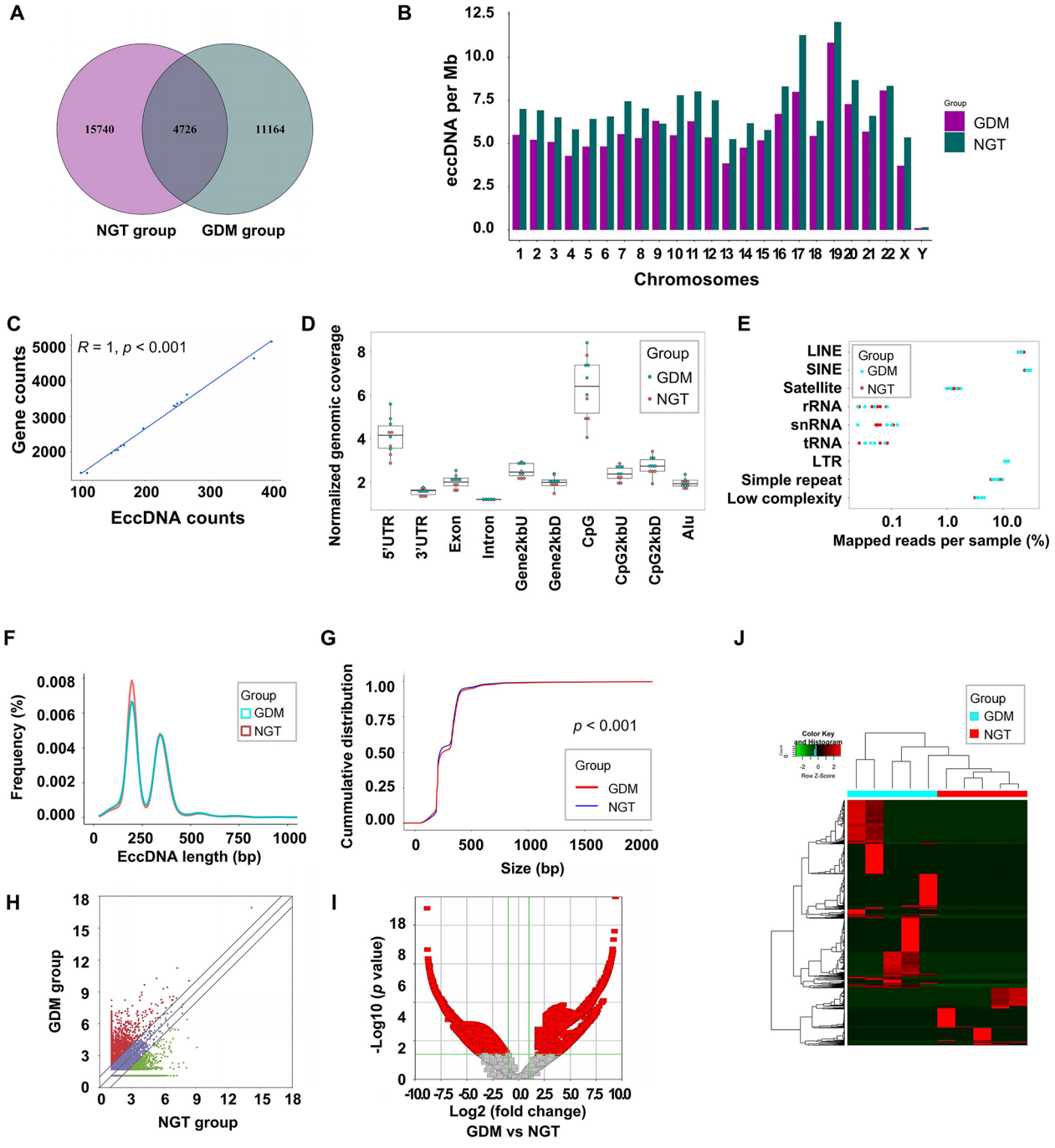

Fig. 2 The profiles of eccDNAs in women categorized by future GDM development. **A** Venn diagram showing the overlap of eccDNAs between GDM and NGT groups, with a total of 4,726 shared eccDNAs. **B** Bar graph depicting the frequency of eccDNAs per megabase across all chromosomes, differentiated between GDM and NGT groups, indicating genomic distribution. **C** Scatter plot illustrating a significant correlation between the counts of coding genes and eccDNAs ($p < 0.001$), suggesting a potential regulatory or structural relationship. **D** Box plots showing the distribution of eccDNAs in different genomic regions (such as 5'UTR, 3'UTR, exonic, intronic, and intergenic) for both GDM and NGT groups, comparing the genomic context of eccDNA localization. **E** Stacked bar chart representing the proportion of eccDNA mapping to various repeat classes, including LINEs, SINEs, satellite DNA, tRNA, snRNA, LTR, simple repeats, and low complexity regions, for each group, highlighting the diversity of eccDNA origins. **F** Distribution curve of eccDNA length, with separate curves for GDM and NGT groups, showing the frequency of eccDNAs at various lengths, indicating differences in eccDNA size distribution. **G** Cumulative frequency curve for eccDNA size, comparing GDM and NGT groups with a significant size variation marked by a *p*-value of less than 0.001, suggesting distinct physical properties of eccDNAs between groups. **H** Scatter plot comparing eccDNA counts in the GDM group against the NGT group, illustrating individual variability and group trends. **I** Volcano plot showing differential abundant of eccDNAs between GDM and NGT groups, marked by log-fold changes and *p*-values, to identify significantly upregulated or downregulated eccDNAs. **J** Heatmap with hierarchical clustering of eccDNA features distinguishing GDM from NGT samples. eccDNA: extrachromosomal circular DNA, GDM: gestational diabetes mellitus, NGT: normal glucose tolerance

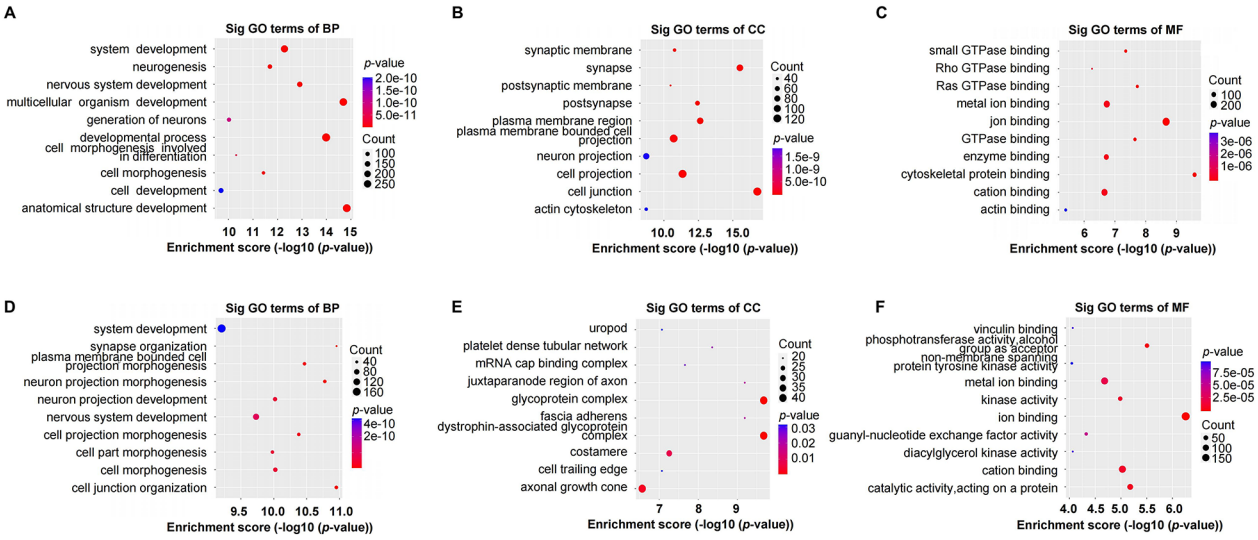

Fig. 3 GO analyses of eccDNAs based on future GDM development. **A–C** Display significant GO terms for increased eccDNAs across BP, CC, and MF, respectively. **D–F** Similar displays for decreased eccDNAs across BP, CC, and MF, detailing enrichment scores and gene counts. 'Sig' stands for 'Significant', indicating GO terms with p-values less than 0.05. GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function

Table 2 The significantly enriched GDM-related signaling pathways for the altered eccDNAs

Term	Regulation	Count	*p*-value	Enrichment score
Protein digestion and absorption	Up	10	0.001	2.91
Glycosphingolipid biosynthesis—ganglio series	Up	4	0.002	2.81
Rap1 signaling pathway	Up	16	0.003	2.48
cGMP-PKG signaling pathway	Up	13	0.006	2.20
Vascular smooth muscle contraction	Up	9	0.026	1.58
Steroid biosynthesis	Up	3	0.032	1.49
Rap1 signaling pathway	Down	13	0.004	2.38
Glycerolipid metabolism	Down	6	0.005	2.33
Phospholipase D signaling pathway	Down	10	0.005	2.29
cAMP signaling pathway	Down	12	0.007	2.16
Hippo signaling pathway	Down	10	0.008	2.09
MAPK signaling pathway	Down	14	0.008	2.08
AMPK signaling pathway	Down	8	0.018	1.75
PI3K-Akt signaling pathway	Down	16	0.020	1.70
Insulin resistance	Down	7	0.027	1.58
cGMP-PKG signaling pathway	Down	9	0.035	1.46
Ras signaling pathway	Down	11	0.041	1.39

Term: the name of the pathway, Count: the count of the chosen eccDNAs directly associated with the listed pathway, *p*-value: the enrichment *p*-value of the pathway using Fisher exact test, Enrichment score: the enrichment score value of the pathway, it equals "−log10 (*p*-value)"

The Integrative Genomics Viewer (IGV) screenshot shows a lower eccDNA PRDM16^{circle} abundance in the GDM group than in the NGT group (Fig. 4A). Specific primers targeting the junction sites were used for outward PCR, and Sanger sequencing verified the circular structure and junction sites of PRDM16^{circle} (Fig. 4B). Gel electrophoresis images displaying eccDNA PRDM16^{circle} are presented (Fig. 4C, Additional file 3: Fig. S1). Furthermore, qPCR analysis of sequenced samples confirmed a significant reduction in PRDM16^{circle} presence in the GDM group, corroborating the eccDNA-seq results ($p<0.001$; Fig. 4D). Moreover, these findings were validated in a new cohort consisting of 140 pregnant women ($p<0.001$; Fig. 4E).

Predictive effect of plasma eccDNA PRDM16^{circle} on GDM

To evaluate the predictive impact of plasma eccDNA PRDM16^{circle} and various clinical parameters on GDM during the 11–13 weeks of gestation, a forest plot was employed to visually depict the association strengths between these variables and the risk of developing GDM (Fig. 5). This plot illustrates odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for factors such as gestational age at sampling, maternal age,

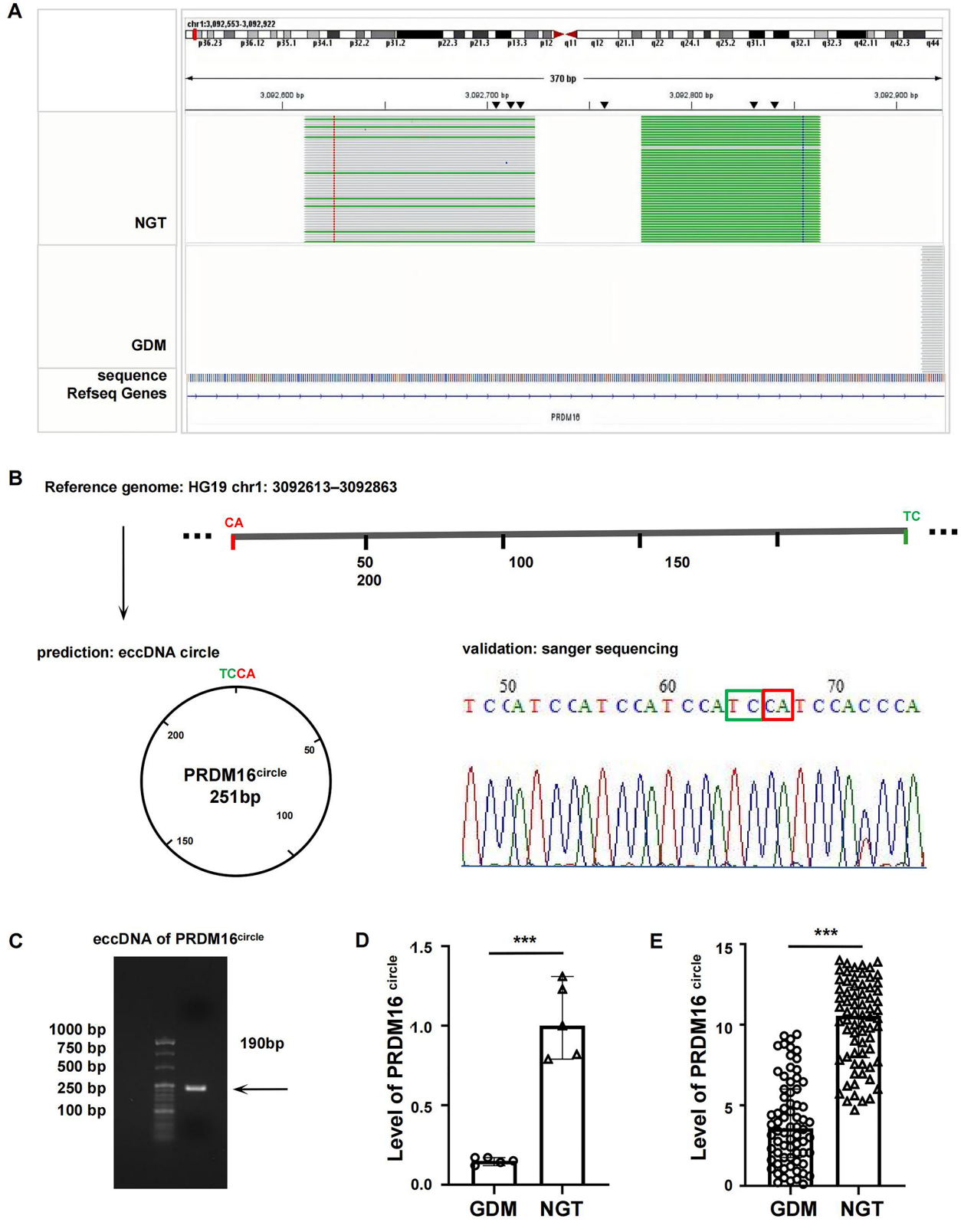

Fig. 4 Plasma eccDNA PRDM16^{circle} level is decreased in women who developed GDM later. **A** Integrative Genomics Viewer (IGV) depicted for eccDNAs derived from the *prdm16* gene. **B** Visualization of eccDNA PRDM16^{circle} at Chr1:3092613–3092863. Junction site of eccDNA PRDM16^{circle} identified as TCCA. **C** Gel electrophoresis validation of PRDM16^{circle}. **D** qPCR validation of PRDM16^{circle} level in 10 sequenced samples. **E** qPCR validations of eccDNA PRDM16^{circle} level in plasma obtained from 70 women who developed GDM compared to 70 NGT women. ***$p < 0.001$. GDM, gestational diabetes mellitus; NGT, normal glucose tolerance

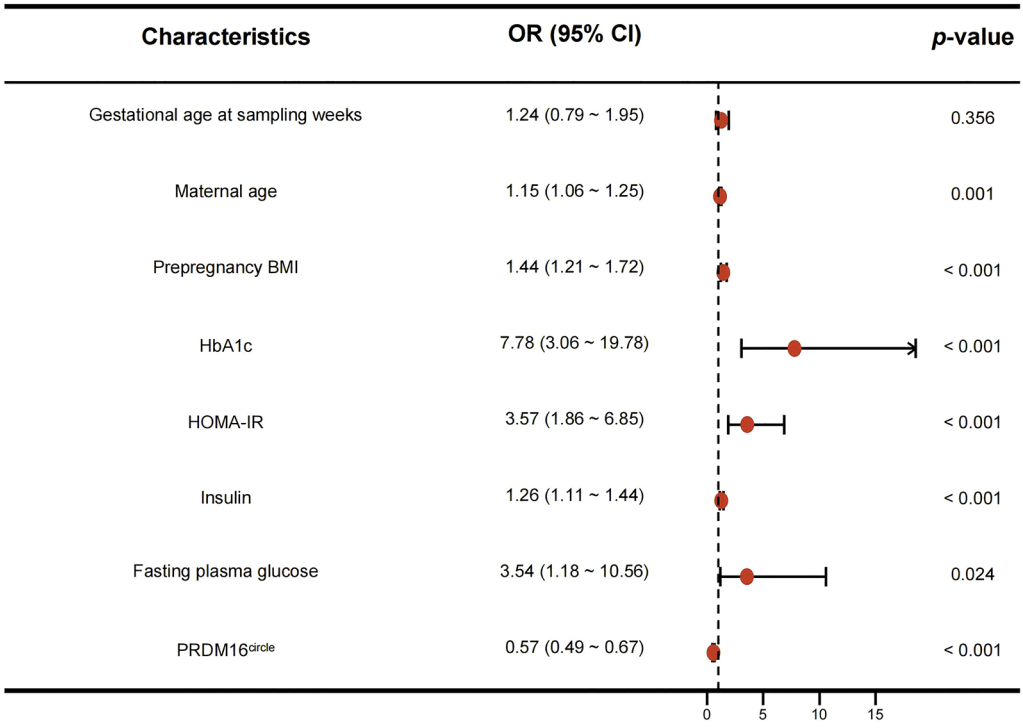

Fig. 5 Forest plot of predictive factors for GDM in early pregnancy. This forest plot illustrates the ORs and 95% CIs for various predictive factors linked to GDM risk. Each line represents a different predictor analyzed in the study, including gestational age at sampling, maternal age, pre-pregnancy BMI, HbA1c, HOMA-IR, insulin levels, fasting plasma glucose, and PRDM16^{circle}. GDM, gestational diabetes mellitus; NGT, normal glucose tolerance; OR, odds ratio; CI, confidence interval; BMI, body mass index; HbA1c, glycated hemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance

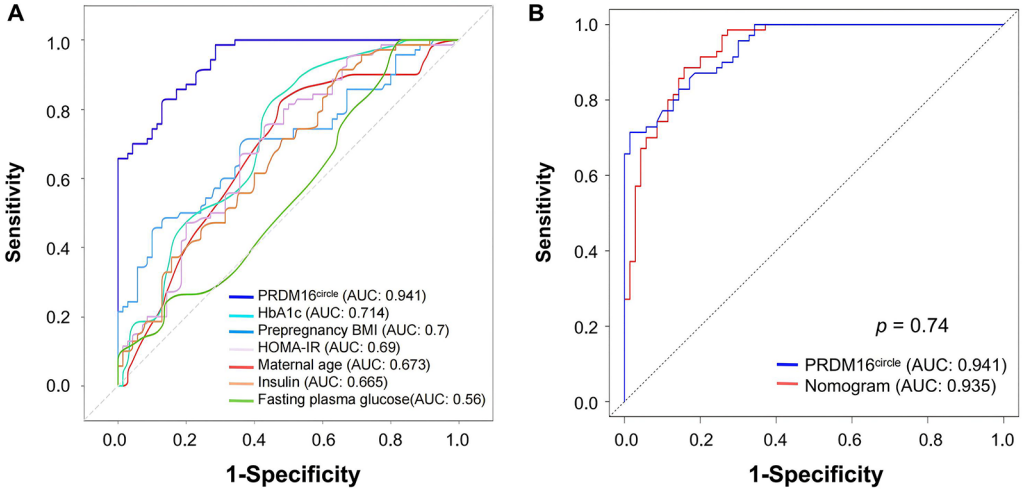

Fig. 6 ROC curves for various predictors of GDM. **A** ROC curves for the capacity of the plasma eccDNA PRDM16^{circle} and other clinical parameters to differentiate GDM patients from NGT subjects. **B** The AUC showed no significant difference between the nomogram model and the eccDNA PRDM16^{circle} predictive model ($p=0.74$). ROC, receiver operating characteristic; AUC, area under the curve; GDM, gestational diabetes mellitus; NGT, normal glucose tolerance

prepregnancy BMI, levels of HbA1c, HOMA-IR, insulin, fasting plasma glucose, and PRDM16^{circle} levels. These findings suggest that lower abundance of PRDM16^{circle} is associated with an increased risk of GDM (OR=0.57, 95% CI 0.49–0.67, $p<0.001$). Conversely, higher levels of HbA1c, maternal age, prepregnancy BMI, HOMA-IR, insulin, and fasting glucose significantly contribute to the risk of GDM. Furthermore, the ROC curves illustrate a range of diagnostic effectiveness, with eccDNA PRDM16^{circle} showing the highest AUC (AUC=0.941; 95%CI 0.907–0.975; $p<0.001$; Fig. 6A), indicating superior diagnostic accuracy compared to the other variables such

as HbA1c, prepregnancy BMI, HOMA-IR, age, insulin, and fasting plasma glucose (Fig. 6A).Then, four variables were selected to construct the nomogram model through a combination of group difference analysis, univariate logistic regression, and LASSO (Additional file 4: Fig. S2). The corresponding score of each predictor was displayed in the nomogram model (Additional file 5: Fig. S3) A Sobol sensitivity analysis of these variables is shown in Additional file 6: Fig. S4, and the AUC of the nomogram logistic regression model was 0.935, with a 95% CI of 0.896–0.974 (Fig. 6B). There was no significant difference in the AUC between this model and the predictive model of eccDNA PRDM16^{circle} (p=0.74), indicating that both models have comparable predictive performance despite slight variations in their AUC values.

Discussion

In this study, Circle-seq analysis was utilized to perform studies on molecular characterization, distribution, and biological-functional of plasma eccDNA at 11–13 weeks' gestation in women who later developed GDM compared to healthy pregnant women. This analysis successfully established a novel eccDNA profile for the women who developed GDM. In the GDM group, compared to NGT subjects, 1289 eccDNAs showed significant upregulation while 928 showed significant downregulation. A novel eccDNA, named PRDM16^{circle}, was selected for further validation of the Circle-Seq data based on the fold change value, p-value, gene locus, and potential molecular mechanisms implicated in GDM. Validation procedures including outward PCR, Sanger sequencing, and qPCR confirmed consistency with the Circle-seq data. qPCR amplification of plasma samples from 70 women who later developed GDM and 70 NGT subjects showed significantly decreased of PRDM16^{circle} in the GDM group, indicating its high predictive value in distinguishing GDM from NGT (AUC=0.941, p<0.001). This indicates that plasma eccDNA PRDM16^{circle} could be an effective biomarker for predicting GDM at 11–13 weeks' gestation. Early identification of women at risk for GDM during pregnancy allows for targeted prevention strategies, including lifestyle modifications. Successfully preventing GDM can substantially reduce both the immediate and long-term negative effects for both mothers and their children.

As biomarkers, eccDNA presents several advantages over linear cell-free DNA (cfDNA). The closed circular structure of eccDNA makes it resistant to exonuclease digestion and more stable than linear DNA [13, 17, 19]. Rolling-circle amplification of eccDNAs eliminates the need for site-specific primers, commonly used for detecting linear cfDNA. This method supports the impartial detection of circular DNA throughout the genome without bias related to its original locations [23]. eccDNA in plasma emerges as an ideal source of potential biomarkers, not only for various cancers but also for disorders such as type 2 diabetes, fetal growth restriction (FGR), and systemic lupus erythematosus (SLE) [16, 18, 20, 24–26].

EccDNA plays a crucial role in activating innate immunity and acts as a potent stimulant for this system. Cleavage of eccDNAs into linear fragments results in a loss of their potent immunostimulatory capability, indicating the importance of their circular form and interaction with the cytosolic DNA sensor Sting for immune response [27]. In dendritic cells and macrophages, eccDNA triggers greater cytokine production (including type I interferons (IFN-α, β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α)) than linear DNA fragments of similar size, effectively enhancing immune response activation [27]. This demonstrates the sensing pathways of eccDNA in immune responses and its potential clinical significance. Inflammation during early pregnancy is linked to a higher risk of GDM later on [28, 29]. Higher early pregnancy inflammatory markers correlate with greater insulin resistance risk, leading to GDM [28, 29]. Notably, GDM is also commonly linked to increased oxidative stress, which not only intensifies inflammatory responses but also correlates with increased insulin resistance [1]. Furthermore, inflammatory factors can affect the regulation of glucose metabolism by inhibiting tyrosine phosphorylation of insulin receptor substrates and reducing insulin receptor activity [30, 31]. Our findings reveal a significant presence of eccDNA in the plasma of GDM and NGT. The differentially abundant eccDNA, upon GO and KEGG analysis, enriches several GDM-related signaling pathways including the Rap1 signaling pathway [32], MAPK signaling pathway [33], PI3K-Akt signaling pathway [33, 34], and Insulin resistance signaling pathway [35], etc. (Table 2).

Our findings demonstrated that eccDNA PRDM16^{circle} is significantly decreasedin the plasma of women during early pregnancy who later develop GDM. Nonetheless, the precise biological function of PRDM16^{circle} in the development of GDM requires additional investigation. eccDNA, a class of small circular DNA, is capable of replicating independently from chromosomes and may host genes that are expressed [21]. These molecules can modulate the expression of essential genes through their interactions with the main genome, thereby impacting cellular functions and the progression of disorders [36, 37]. Additionally, eccDNAs possess transcriptional activity, influencing phenotypic variation by hosting genes that can be expressed in both full-length and truncated forms [21, 38]. They play crucial roles in gene expression regulation, genomic instability, signaling communication, and immune responses [13, 21, 38, 39]. Consequently, abnormalities in eccDNA can serve as pathological triggers, initiating and advancing various diseases.

PRDM16, a critical transcription factor, has been shown to be involved in the regulation of several metabolic processes, primarily glucose homeostasis, oxidative stress and lipid metabolism [40, 41]. Through the PRDM16-GTF2IRD1 complex, PRDM16 curbs adipose tissue fibrosis, reducing diet-induced glucose tolerance and insulin resistance [42]. Furthermore, extended stability of the PRDM16 protein mitigates the effects of diet-induced obesity, glucose intolerance, insulin resistance, and dyslipidemia in mice [43]. Interestingly, hypermethylation of PRDM16, as observed in offspring exposed to maternal diabetes in utero, correlates with a higher diabetes risk [44]. In light of PRDM16's critical role in adipose tissue metabolism, particularly through its involvement in the browning and thermogenesis of adipocytes, it presents a promising target for obesity and diabetes mellitus (DM) therapies. The stabilization of PRDM16 is underscored as having therapeutic potential not only in managing obesity but also in treating diabetes, through the influence of drugs such as GLP-1 agonists, which are known to affect PRDM16-related pathways [40, 41]. Specifically, agents like metformin and rosiglitazone have been demonstrated to activate PRDM16 either directly or via intermediates such as AMPK and PPARγ, thereby enhancing adipocyte differentiation and thermogenic capacity [45, 46]. By highlighting these connections, our findings not only reinforce the diagnostic potential of eccDNA PRDM16^{circle} in early GDM prediction but also may provide a crucial molecular target for developing new therapeutic strategies. More research is needed to directly assess eccDNA PRDM16^{circle}'s effects.

As shown in Fig. 5, our study identified an association between HbA1c and the future development of GDM (OR 7.78, $p<0.001$). This is consistent with the existing literature that underscores the predictive value of HbA1c for GDM [47]. However, it is noteworthy that some studies, such as Immanuel et al. (2020), found that early pregnancy HbA1c had limited capacity to prognosticate GDM when assessed across a large cohort of 869 pregnant women from nine European countries [48]. This discrepancy might be due to differences in study populations, diagnostic criteria, and HbA1c measurement techniques. Similarly, our findings on maternal age (OR 1.15, $p=0.001$) and prepregnancy BMI (OR 1.44, $p<0.001$) align with the broader evidence base, which consistently identifies these factors as significant predictors of GDM. The associations we observed are in agreement with previous studies demonstrating that higher maternal age and increased BMI are significant risk factors for GDM [47]. Studies have shown that markers such as ANGPTL4, BAFF, and APRIL are relevant in the context of GDM. Integrating these markers with PRDM16^{circle} could potentially improve the predictive accuracy and provide a more comprehensive risk assessment model for GDM.

In this study, maternal age was identified as a significant predictor for GDM, emphasizing its association with increased GDM risk due to aging. Additionally, our findings on eccDNA PRDM16^{circle} align with literature emphasizing eccDNA's role in aging and age-related diseases [15]. For instance, studies have demonstrated nuclear retention of circular DNA in yeast, suggesting a similar mechanism may occur in human aging [49, 50]. Further research has linked eccDNA accumulation with age-related conditions such as ALS [51] and osteoporosis [52], reinforcing the relevance of eccDNA in GDM pathology. These insights underscore the importance of considering age in predicting GDM and in research concerning eccDNA.

This study found no significant differences in the AUC between the predictive model of PRDM16^{circle} and the nomogram model. This indicates that the additional variables incorporated into the nomogram model did not significantly enhance the model's predictive power for GDM. The likely explanation is that these clinical parameters are not strong predictive variables. Indeed, adding extra variables does not always improve model performance, especially if these variables do not contribute additional unique information [53]. Moreover, given the limitations of this study's single-center, small-sample design, further verification through multi-center, large-scale studies is necessary to affirm PRDM16^{circle} in plasma as an effective biomarker for early GDM prediction. The limited dataset could lead to an overestimation of the model's predictive accuracy. Future model development should focus on optimizing sample size and carefully selecting predictive factors to balance model complexity with generalizability. Although altered plasma eccDNAs between the GDM and NGT groups at 11–13 weeks' gestation were identified, and the sample size was increased to validate the high predictive utility of PRDM16^{circle} in distinguishing between these groups, the study remains preliminary and has several limitations. Initially, the short treatment time of exonuclease V on plasma DNA samples might result in incomplete removal of linear DNA [22]. Furthermore, the specific molecular mechanisms leading to the decrease in PRDM16^{circle} in GDM were not further investigated. The formation and presence of eccDNA are influenced by various factors, including DNA damage repair, homologous recombination, microhomology-mediated end joining, and chromatin organization [54]. Additionally, DNA replication and transcription processes are believed to generate single-stranded DNA-based eccDNA, possibly involving replication slippage or R-loop formation [54, 55]. Future research should focus on evaluating PRDM16 transcript levels and potential mechanisms such as double-strand breaks, break-fusion-bridges (BFB) cycles or replication slippage in the PRDM16 gene within placental or other tissues.

In conclusion, for the first time, we report that GDM patients exhibit a unique eccDNA pattern compared to NGT at 11–13 weeks of gestation, exploring their characteristics and biological functions. PRDM16^{circle} in plasma is decreased in women who develop GDM but demonstrates significant predictive ability for differentiating between GDM and NGT. It may serve as an early biomarker for detecting pregnant women at increased risk of GDM, potentially facilitating timely interventions that may decelerate the progression of GDM and enhance maternal-infant health outcomes. In addition, various eccDNAs in pregnant women remain understudied. Exploring these eccDNAs using cutting-edge tools will reveal new mechanisms underlying GDM biology.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12933-024-02381-1 .

Additional file 1.

Additional file 2.

Additional file 3: Figure S1. Full uncropped gel image. This image presents the full, uncropped gel utilized in the study, including: Lanes 1–3: Samples from other experiments not related to this study. These lanes serve to demonstrate the gel's broader usage but are not discussed in the current paper. Lane 4: Sample used in this research, showing the amplified product of eccDNA PRDM16circle.

Additional file 4: Figure S2. Analysis of feature selection using LASSO method and determination of optimal regularization parameter. A Feature selection was conducted using the LASSO method. The optimal regularization parameter (λ) was determined based on the criterion of minimal mean squared error obtained through tenfold cross-validation. B The plot includes a vertical line that marks the log(λ) value corresponding to the optimal λ, at which precisely seven features retain non-zero coefficients. LASSO: Least Absolute Shrinkage and Selection Operator.

Additional file 5: Figure S3. Nomogram for predicting GDM risk. This nomogram combines maternal age, pre-pregnancy BMI, and HbA1c levels to estimate GDM risk. The value of PRDM16^{circle} was detected by qRT-PCR, and the results are presented as fold change ($2^{-\Delta\Delta Ct}$) without log2 transformation. Each factor is assigned to points which are summed to calculate a total risk score, shown on the 'Risk' axis. The model also assesses the impact of the novel predictor PRDM16^{circle}. This tool aids clinicians in quantifying GDM risk in pregnant women.

Additional file 6: Figure S4. Sobol sensitivity analysis of parameters influencing GDM development. This figure illustrates the Sobol Total Index for parameters influencing the development of GDM. PRDM16^{circle} shows the highest sensitivity index, indicating its significant role, followed by HbA1c, Prepregnancy BMI, and Age.

Author contributions

Jin Wang: Performed experiments and wrote the manuscript. Pengyu Huang: Performed data analysis and prepared the manuscript. Fei Hou: Performed experiments. Dongdong Hao and Wushan Li: Managed and followed up with the study cohort. Hua Jin: Designed the study and revised the manuscript.

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Availability of data and materials

The Circle-seq data are available in the Genome Sequence Archive (GSA, https://ngdc.cncb.ac.cn/gsa/) under accession number HRA007356.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The research protocol conformed to the ethical principles of the Declaration of Helsinki and received approval from the local ethics committee (No. IRB KY-23–57). All participants provided written informed consent.

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