Original Research

Analysis, validation, and discussion of key genes in placenta of patients with gestational diabetes mellitus

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Impact statement

The genomic profile of the placenta shares similarities with childhood cancer in terms of mutation load and mutational patterns, suggesting the changes of key genes in placenta may be closely related to some gestational complications, such as gestational diabetes mellitus (GDM). By analyzing two datasets, we screened 20 key genes that were confirmed in placental tissues. We further validated these genes in high-glucose-treated HTR8/SVneo cells, providing evidence for a causal relationship that has been the subject of much debate. Correlation analysis with blood glucose levels of GDM patients suggested that some of these key genes have the potential to be used as biomarkers. Some of these selected key genes are of great research value, and their mechanisms will be further explored in future studies.

Abstract

Gestational diabetes mellitus (GDM) is a common complication during pregnancy, which can have harmful health consequences for both the mother and the fetus. Given the placenta's crucial role as an endocrine organ during pregnancy, exploring and validating key genes in the placenta hold significant potential in the realm of GDM prevention and treatment. In this study, differentially expressed genes (DEGs) were identified from two databases, GSE70493 and PRJNA646212, and verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in placenta tissues. DEGs expression was detected in normal or high-glucose-treated HTR8/SVneo cells. We also investigated the relationship between DEGs and glucose levels in GDM patients. By selecting the intersection of the two databases, we screened 20 DEGs, which were validated in GDM patients. We observed an up-regulation of SLAMF, ALDH1A2, and CHI3L2, and a down-regulation of HLA-E, MYH11, HLA-DRB5, ITGAX, GZMB, NAIP, TMEM74B, RANBP3L, PAEP, WT-1, and CEP170. We conducted further investigations into the expression of DEGs in HTR8/SVneo cells exposed to high glucose, revealing a significant upregulation in the expression of SERPINA3, while the expressions of HLA-E, BCL6, NAIP, PAEP, MUC16, WT-1, and CEP170 were decreased. Moreover, some DEGs were confirmed to have a positive or negative correlation with blood glucose levels of GDM patients through correlation analysis. The identified DEGs are anticipated to exert potential implications in the prevention and management of GDM, thereby offering potential benefits for improving pregnancy outcomes and long-term prognosis of fetuses among individuals affected by GDM.

Keywords: Gestational diabetes mellitus, bioinformatics, placenta, trophoblast, translational medicine, key genes

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Introduction

Gestational diabetes mellitus (GDM) refers to a condition of impaired glucose tolerance that emerges during pregnancy and can result in poor pregnancy outcomes for both mothers and fetuses. GDM mothers are more likely to suffer from gestational hypertension and pre-eclampsia during pregnancy and are at an elevated risk of developing type 2 diabetes in the long term.[1–3] Their babies are prone to suffer from macrosomia, preterm birth, neonatal hypoglycemia, and so on.[3–5] The global prevalence of GDM varies between 9% and 26%, with an average prevalence of approximately 18%,[6] significantly impacting the socioeconomic burden on societies.

Gene chip technology and sequencing technology have undergone rapid development, providing unprecedented convenience for researchers in the search for novel biomarkers and potential therapeutic targets for many diseases.[7,8] Many scholars have investigated the pathogenesis of GDM and sought therapeutic agents for GDM through bioinformatics methods.[9–12] For instance, potential biomarkers for GDM prediction and diagnosis have been identified by identifying several differential metabolites.[10] Furthermore, the

results of transcriptomic profiling of human placenta at the single-cell level by Yang *et al.* help to elucidate the molecular mechanisms of GDM.[12] These findings are encouraging because they have greater clinical relevance, are more easily translated into practical applications, and can provide directions for basic research.

However, caution must be exercised when applying these results to the study of disease occurrence and progression, as the differentially expressed genes (DEGs), miRNAs, lncRNAs, metabolites, and other factors identified through gene chip or sequencing technology may only reflect what is found after the disease has occurred. It is difficult to determine whether these differential expressions are the cause of the disease or simply a result of it. For example, Liu *et al.* found that cystathionine gamma-lyase (CSE) O-linked β-N-acetylglucosamine (O-GlcNAc) and H2S production increased in pre-eclamptic placenta,[13] but further research into the mechanism revealed a significant association between increased CSE O-GlcNAc and H2S production and insufficient trophoblast sysncytialization, suggesting a compensatory response of the placenta to maintain a healthy pregnancy. Thus, mistaking this change as a cause and intervening may actually worsen the disease.

Given the placenta's role as a vital endocrine organ during pregnancy, its importance in the influence of GDM on the mother and fetus is reflected in two aspects: first, abnormal placental function may cause the onset of GDM[14]; second, GDM may lead to abnormal placental function, which can affect maternal–fetal material exchange.[15,16] For instance, upregulation of interleukin (IL)-15 in the placenta can alter trophoblast function and promote the occurrence of GDM.[17] In addition, studies have shown that GDM patients have a deficiency in placental fatty acid transporters,[18,19] which can lead to long-term neurodevelopmental abnormalities in their offspring[2] due to a reduced acquisition of long-chain polyunsaturated fatty acids that are essential for early-life neural formation. It is noteworthy that alterations in the placenta can act as both a causative factor for GDM and as a consequence of alterations in the body's environment after the onset of GDM, such as insulin resistance or a high-glucose environment. Some of these changes may be compensatory, and pathway studies of this part may find new therapeutic targets to improve maternal and offspring health in GDM.[13,18] Therefore, identifying and verifying key genes in the placenta could be of great interest for both prevention and treatment.

In this study, we obtained two datasets on GDM from Gene Expression Omnibus (GEO) and the European Nucleotide Archive (ENA), respectively. We conducted a screening of DEGs and identified 20 common DEGs by taking the intersection of the two datasets. We verified the expression differences of these 20 DEGs in placenta from women diagnosed with GDM and analyzed the correlations between the expressions of these DEGs and early pregnancy blood glucose levels, oral glucose tolerance test (OGTT) results, and late pregnancy blood glucose levels in GDM patients and normal women. Furthermore, to investigate whether the DEGs were a result of the high-glucose environment caused by GDM, we established a cellular model using HTR8/SVneo cells exposed to high glucose and verified the expressions of these genes in the model. The identified DEGs hold potential implications for the prevention and treatment of GDM, which can be beneficial for the pregnancy outcome and long-term prognosis of fetuses in GDM patients.

Materials and methods

Data processing of GSE70493

The dataset GSE70493, obtained from the GEO database, was used in this study. It was generated using the Affymetrix Human Transcriptome Array 2.0, which incorporates transcript (gene) version information. The dataset included 63 placental tissue specimens, with 32 obtained from women diagnosed with GDM and 31 obtained from pregnant women without GDM (normal pregnant women). The probe-level data (CEL files) were imported into the R programming environment using the Oligo package.[20] Subsequently, background correction, normalization, and expression calculation were performed using the robust multi-array average (RMA) algorithm.[19] Quality control was performed using the ArrayQualityMetrics package,[21] and batch effects were removed using the sva package. Limma package[22] was used to identify DEGs, and only DEGs with a P value of less than 0.05 were chosen for subsequent analysis.

Data processing of PRJNA646212

The dataset PRJNA646212, generated by Illumina Hiseq 4000, was obtained from the ENA database. This dataset consisted of four placental samples from GDM patients and four from control patients. Trim_galore (version 0.6.6) and Cutadapt (v3.4 with Python 3.9.4) were used for data filtering. Bases with low quality were removed first, then the adapters at the 3′ were removed. After filtering the original data, the clean data obtained by quality control verification data were qualified for downstream analysis, and the transcript expression matrix was obtained by RNA-seq analysis with Hisat2 and FeatureCounts. The DESeq2[23] package was used to identify DEGs, and only DEGs with a P value < 0.05 and an absolute log2-fold change ($|\log2FC| > 1$ were selected for further analysis.

Intersection processing

A Venn diagram was generated using Evenn (http://www.ehbio.com/test/venn/#/)[24] to visualize the overlapping DEGs. The Search Tool for the Retrieval of Interacting Genes (STRING, version 11.5, https://cn.string-db.org/)[25] database was used to analyze the interactions among the DEGs and established a Protein–protein interaction (PPI) network. All intersectant DEGs were evaluated by gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses with the ClusterProfiler[26] package in R and P value < 0.05 served as the threshold.

Participants and samples

We recruited 28 pregnant women from Tongji Hospital who received antenatal examinations from early pregnancy to delivery. Among the participants, 14 women were diagnosed with GDM, while the remaining 14 women had

uncomplicated pregnancies. All participants underwent a 75-g OGTT between 24 and 28 weeks of gestation and had no other pregnancy complications. Written informed consent was obtained from all participants, and the research was approved by the Ethics Board of Tongji Hospital.

One cubic centimeter of placental tissue from the maternal side was collected at the time of cesarean delivery. To prevent RNA degradation, the tissues were rinsed with cold phosphate-buffered saline (PBS) and immediately stored in RNA later (Thermo Fisher Scientific, USA) for transport. RNA extraction was performed on the same day.

Cell culture and treatment

The HTR-8/SVneo cell line obtained from Servicebio Technology (Wuhan, China) was cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO2. The high-glucose HTR8/SVneo cells model had a glucose concentration of 25 mmol/L.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from all placental tissues and HTR8/SVneo cells by TRIzol reagent (Takara, Japan), and HiScript® II Q RT SuperMix for qPCR (Vazyme, China) was used for cDNA synthesis of mRNA. The expression of all DEGs was normalized using ACTB as a reference gene.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was conducted using the Bio-rad CFX CONNECT Real-Time System (Bio-rad, Hercules, California, USA). The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the control samples with ACTB as the reference. The primer sequences used for RT-qPCR can be found in the supplementary materials of the study.

Statistical analysis

Continuous variables were reported as mean ± standard deviation (SD) for all analyses. Statistical analyses were conducted using GraphPad Prism 9.0 software. Student's *t*-test or non-parametric tests were employed to compare two independent groups, depending on the distribution of the data. Pearson's correlation analysis or Spearman's correlation analysis was performed to investigate the relationship between expressions of DEGs and the blood glucose levels of pregnant women. The data from each experiment were derived from at least three independent experiments. Statistical significance was set at a two-side *P* value less than 0.05.

Results

Analysis of GSE70493 and PRJNA646212

The analysis of GSE70493 is depicted in Figure 1, showcasing the results. A comprehensive total of 1251 DEGs were identified, comprising 656 up-regulated DEGs and 595 down-regulated DEGs, as indicated in the volcano plot (Figure 1(a)). Figure 1(b) shows the heatmap, where the red strip represents GDM samples and the blue strip represents normal samples. Similarly, the analysis results for PRJNA646212 are shown in Figure 1. From this dataset, we obtained a total of 316 DEGs, comprising 79 up-regulated DEGs and 237 down-regulated DEGs (Figure 1(c)). Using the clustering heatmap (Figure 1(d)), we observed significant differences in the expressions of these DEGs between GDM and normal placenta, and the trend of the same group showed consistency. Supplementary Figure 1 presents the GO and KEGG enrichment analysis results for the DEGs identified in both datasets.

Analysis of intersection of two datasets

The intersection of the DEGs obtained from GSE70493 and PRJNA646212 analyses yielded 21 common DEGs, namely HLA-E, CST7, SLAMF7, MEDAG, MYH11, ALDH1A2, HLA-DRB5, BCL6, ITGAX, GZMB, NAIP, NDUFA6, SERPINA3, TMEM74B, RANBP3L, PAEP, CHI3L2, MUC16, WT1, CEP170, and LOC100133286 (Figure 2(a)). LOC100133286 could not be annotated in any database, and therefore, it was removed, leaving us with a final set of 20 DEGs. GO analysis and KEGG pathway analysis were conducted for these 20 DEGs, and the results were shown in Figure 2(b) and (c). The majority of biological processes were associated with immune function, while most cellular components were associated with major histocompatibility complex (MHC) protein and luminal side of the membrane. Peptide antigen binding, endopeptidase inhibitor/regulator activity, and peptidase inhibitor/regulator activity were enriched in the molecular function category. The results of the KEGG pathway analysis included significantly enriched categories such as type 1 diabetes mellitus, graft-versus-host disease, allograft rejection, and antigen processing and presentation. Figure 2(d) depicted a simple protein–protein interaction network among these DEGs established by STRING, suggesting that there was a close relationship between these DEGs.

Validation of 20 DEGs expressions in clinical specimens

Table 1 presents a comprehensive summary of the clinical characteristics of the participants enrolled in the study. The study included a total of 28 pregnant women, with 14 of them diagnosed with GDM. The GDM group had a mean age of 32.50 ± 3.30 years, while the normal group consisted of 14 women with an average age of 30.29 ± 2.49 years. The GDM group exhibited significantly higher plasma glucose levels compared to the normal group ($P < 0.05$). In addition, the pre-pregnancy body mass index (BMI) of the GDM group was 24.95 ± 2.92 kg/m^2, which exhibited a significant increase compared to the normal group.

We verified the expressions of the 20 DEGs in placenta specimens of GDM patients compared with those of normal specimens, and the results are presented in Figure 3. In the placental tissues of GDM patients, the expressions of HLA-E, HLA-DRB5, GZMB, TMEM74B, PAEP, MYH11, ITGAX, NAIP, RANBP3L, WT-1, and CEP, among the DEGs, exhibited significant down-regulation (Figure 3(a)), while the expressions of SLAMF7, ALDH1A2, and CHI3L2 were found to be increased (Figure 3(b)). There were no significant

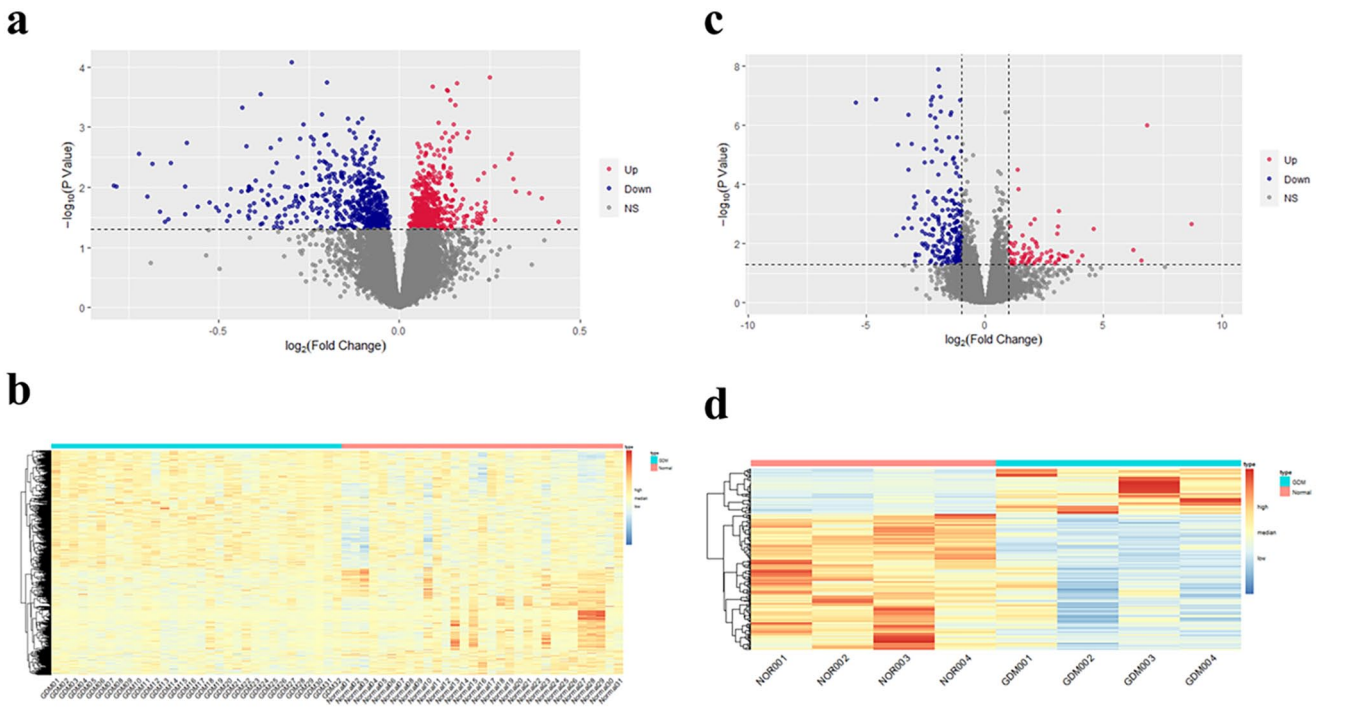

Figure 1. The analysis results of GSE70493 and PRJNA646212: (a) volcano plot of differentially expressed genes (DEGs). Red dots indicate genes that are up-regulated, while blue dots represent genes that are down-regulated. (b) Cluster heatmap of DEGs. The red strip represented GDM samples, and the blue strip represented normal samples. (c) Volcano plot of DEGs. Red dots indicate genes that are up-regulated, while blue dots represent genes that are down-regulated. (d) Cluster heatmap of DEGs. The red strip represented GDM samples, and the blue strip represented normal samples.

differences observed in the expressions of CST7, MEDAG, BCL6, NDUFA6, SERPINA3, and MUC16 between GDM placenta and normal placenta (Figure 3(c)).

Validation of 20 DEGs expressions in high-glucose-treated HTR8/SVneo cells

In this study, a high-glucose HTR8/SVneo cell model was established to examine the expressions of DEGs under this specific condition. Our findings showed that exposure of HTR8/SVneo cells to high glucose resulted in significant decreases in the expressions of HLA-E, MUC16, NAIP, WT-1, BCL6, PAEP, and CEP170, while SERPINA3 expression was increased. No significant differences were observed in the expressions of NDUFA6, TMEM74B, MEDAG, HLA-DRB5, and SLAMF. In addition, the poor melting curves of MYH11, CST7, RANBP3L, GZMB, ALHD1A2, CHI3L2, and ITGAX, coupled with high CT values (>35), suggested that these genes may not be expressed in HTR8/SVneo cells (Figure 4).

In Table 2, it was observed that the expressions of HLA-E, NAIP, PAEP, WT-1, and CEP170 were decreased in HT8/SVneo cells treated with high glucose, which was consistent with the trends in GDM placental tissues, indicating that the observed alterations in the expressions of these genes in GDM placental tissues may be attributed to the high-glucose environment rather than being the underlying cause of GDM. In contrast, the expression changes of SLAMF (up-regulated), HLA-DRB5 (down-regulated), and TMEM74B (down-regulated) were exclusively observed in GDM placental tissues but not in high-glucose-treated HTR8/SVneo cells, suggesting their potential role in the development of GDM. Furthermore, despite the observed alterations in the expressions of MYH11 (down-regulated), ALDH1A2 (up-regulated), ITGAX (down-regulated), GZMB (down-regulated), RANBP3L (down-regulated), and CHI3L2 (up-regulated) in GDM placenta tissues, further investigations are warranted to explore the potential contribution of these genes to the pathogenesis of GDM, considering their limited expression levels in HTR8/SVneo cells.

Correlation analysis between DEGs expressions and blood glucose levels

We carried out the correlation analysis of DEGs expressions and blood glucose levels, including fasting plasma glucose (FPG) in the first trimester, OGTT–0 h, OGTT–1 h, OGTT–2 h, and FPG in the third trimester (Figure 5).

Some DEGs, including HLA-E, MYH11, HLA-DRB5, NAIP, TMEM74B, MUC16, MEDAG, ALDH1A2, PAEP, and WT-1 were confirmed to have certain correlation with blood glucose levels.

DEGs associated with OGTT-0 h blood glucose levels contained HLA-E ($r = -0.5282$, $P = 0.0039$), MYH11 ($r = -0.4172$, $P = 0.0272$), HLA-DRB5 ($r = -0.5300$, $P = 0.0037$), NAIP ($r = -0.3895$, $P = 0.0405$), TMEM74B ($r = -0.4654$, $P = 0.0126$), and MUC16 ($r = 0.3895$, $P = 0.0405$). DEGs associated with OGTT-1 h blood glucose levels contained HLA-E ($r = -0.4558$, $P = 0.0148$), MEDAG ($r = -0.4754$, $P = 0.0106$), ALDH1A2 ($r = 0.4017$, $P = 0.0341$), HLA-DRB5 ($r = -0.4724$, $P = 0.0111$), TMEM74B ($r = -0.5845$, $P = 0.0011$), PAEP ($r = -0.4255$, $P = 0.0240$), and WT-1 ($r = -0.4823$, $P = 0.0093$). DEGs associated with OGTT-2 h blood glucose levels contained

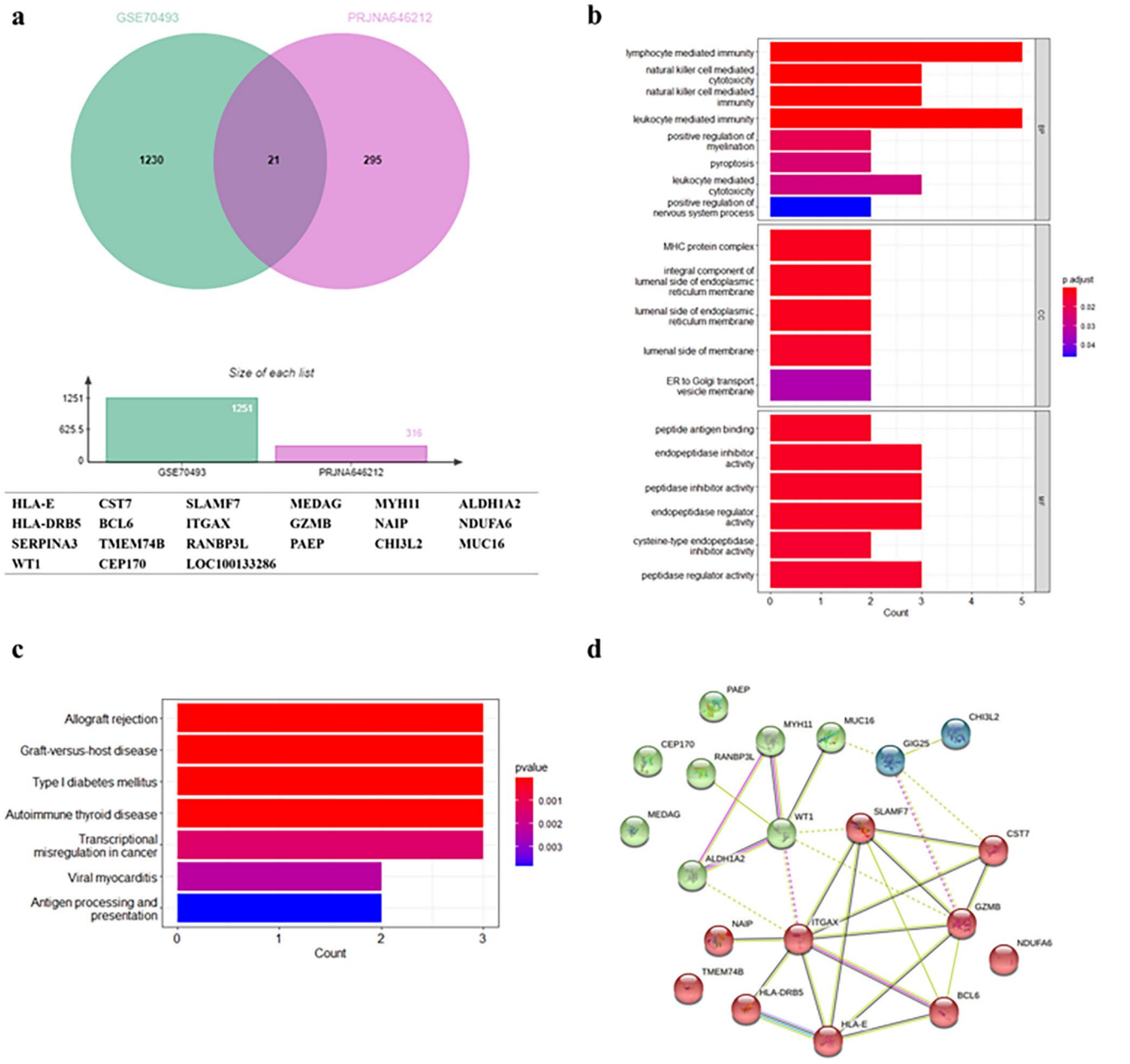

Figure 2. The analysis results of the intersection of GSE70493 and PRJNA646212: (a) the Venn diagram of GSE70493 and PRJNA646212, (b) GO enrichment analysis of the common genes by *P* value, (c) KEGG pathway enrichment analysis of the common genes by *P* value, and (d) protein–protein interaction of common genes.

ALDH1A2 ($r = 0.4255$, $P = 0.0251$), HLA-DRB5 ($r = -0.4691$, $P = 0.0118$), TMEM74B ($r = -0.4215$, $P = 0.0255$), MUC16 ($r = 0.4042$, $P = 0.0329$) and WT-1 ($r = -0.4532$, $P = 0.0154$).

Nevertheless, the analysis revealed no significant correlation between the expressions of the 20 DEGs and FPG levels in the first and third trimesters, suggesting these DEGs might be more related to impaired glucose tolerance than impaired fasting glucose. A summary of the findings regarding these DEGs is presented in Table 2.

Discussion

GDM is a prevalent pregnancy complication with potential adverse health consequences for both mothers and fetuses.[5,27] Extensive research has identified numerous genes that are associated with GDM,[28] highlighting the importance of bioinformatics analysis in GDM researches. When analyzing gene expression data, a single data set may produce a large number of DEGs, but these results can be influenced by study heterogeneity, making it challenging to identify key genes that are truly involved in the disease process. Therefore, intersecting DEGs obtained from multiple datasets can help researchers discover important genes that may play a crucial role in disease development. In our study, we identified 20 DEGs (HLA-E, CST7, SLAMF7, MEDAG, MYH11, ALDH1A2, HLA-DRB5, BCL6, ITGAX, GZMB, NAIP, NDUFA6, SERPINA3, TMEM74B, RANBP3L, PAEP, CHI3L2, MUC16, WT1, and CEP170) that may hold potential

Table 1. Clinical characteristics of GDM and normal groups.

Characteristic	Normal ($n=14$)	GDM ($n=14$)	P value
Maternal age (years)	30.29 ± 2.49	32.50 ± 3.30	0.0557
Gestational age at delivery (days)	268.8 ± 5.12	267.1 ± 5.29	0.4112
Pre-pregnancy BMI (kg/m^2)	22.86 ± 1.48	24.95 ± 2.92	0.0205*
FPG(⩽12weeks) (mmol/L)	4.70 ± 0.42	5.15 ± 0.62	0.0127*
FPG-OGTT (mmol/L)	4.60 ± 0.26	5.61 ± 1.27	0.0065**
1Hr-OGTT (mmol/L)	8.47 ± 0.83	10.34 ± 2.15	0.0055**
2Hr-OGTT (mmol/L)	6.82 ± 0.66	9.08 ± 2.69	0.0052**
FPG(⩾28 weeks) (mmol/L)	4.89 ± 0.71	5.22 ± 0.80	0.2611
Birth weight of newborn (g)	3094 ± 284.1	3321 ± 351.1	0.0703

Clinical characteristics of patients and newborns.
GDM: gestational diabetes mellitus; FPG: fasting plasma glucose; OGTT: oral glucose tolerance test; Hr: hour.
*$P<0.05$, **$P<0.01$.

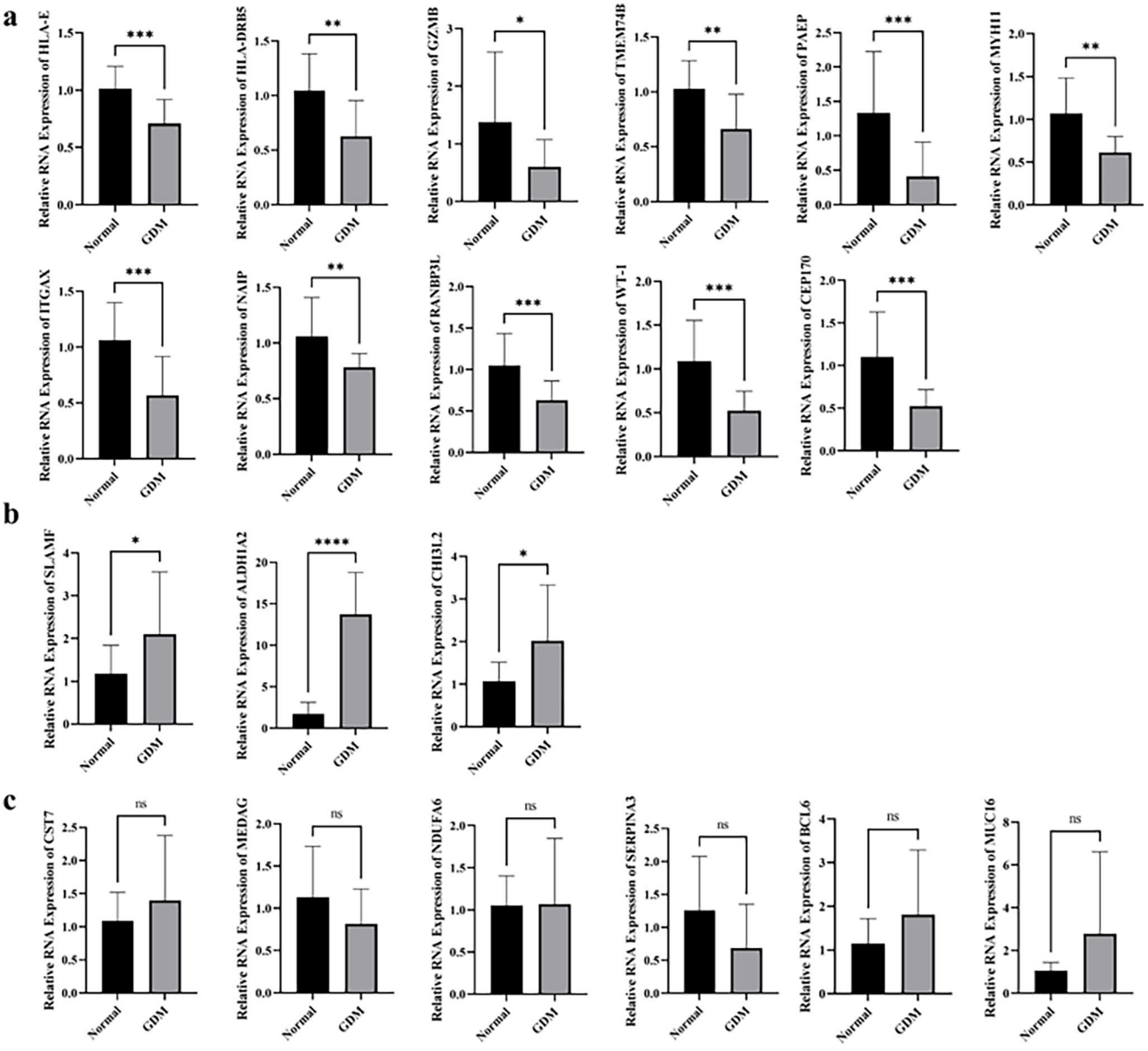

Figure 3. RT-qPCR results of all DEGs in placenta: (a) DEGs which were down-regulated in GDM placenta, (b) DEGs which were up-regulated in GDM placenta, and (c) genes which were not found to differ significantly between GDM and normal placenta.
*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

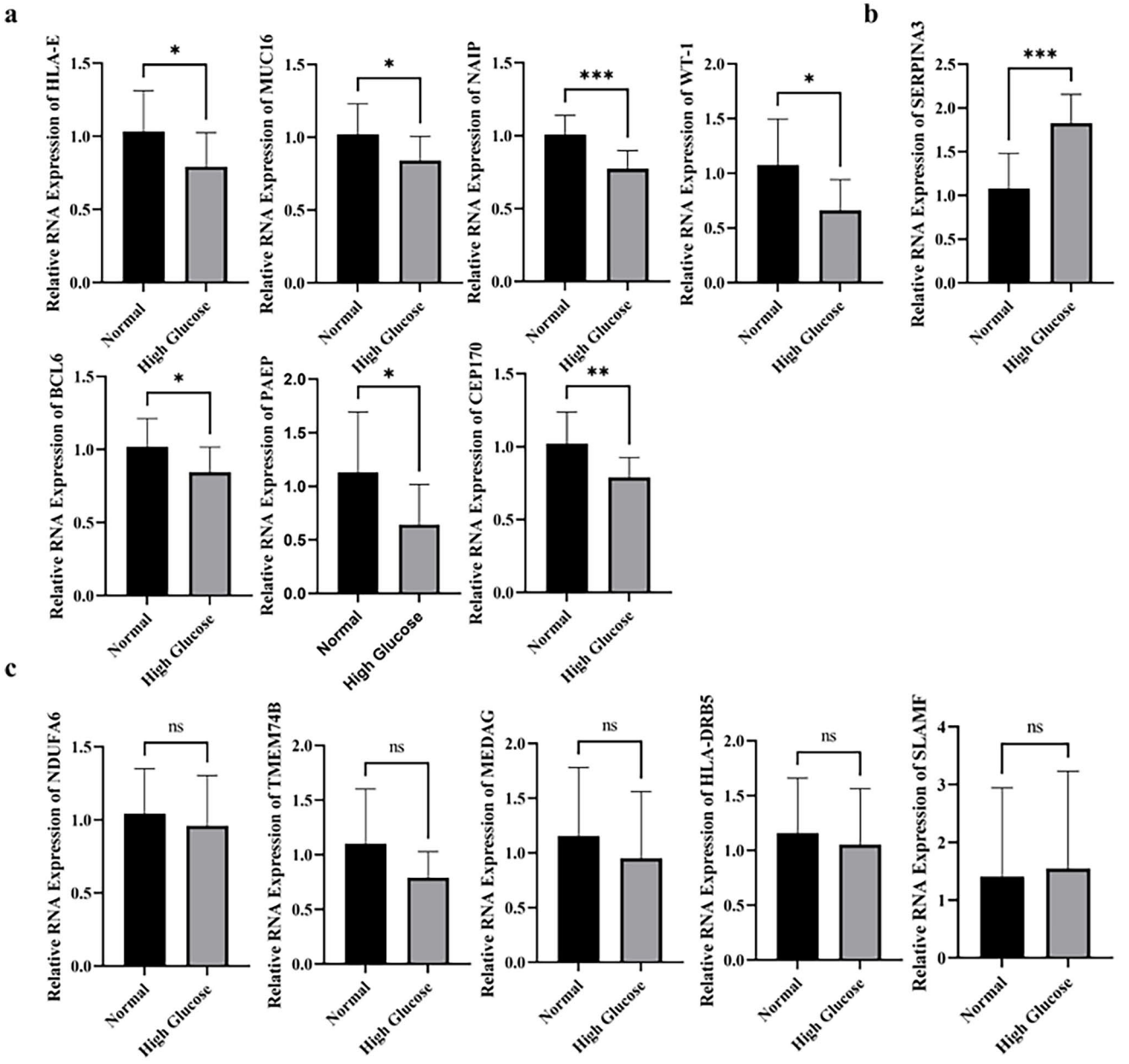

Figure 4. RT-qPCR results of all DEGs in HTR8/SVneo cells: (a) DEGs which were down-regulated in HTR8/SVneo cells exposed to high glucose, (b) SERPINA3 was up-regulated HTR8/SVneo cells treated with high glucose, and (c) genes which were not found to differ significantly between normal HTR8/SVneo cells and treated with high glucose.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

significance in unraveling the underlying mechanisms of GDM pathogenesis by intersecting two datasets. As the differential expression of these selected DEGs was detected in both datasets, their expression changes may be more closely related to the disease and help us narrow down the scope of research without searching blindly. Further research on these genes could be of great value in preventing or treating GDM. To further validate their reliability, we carried out the verification on clinical placenta specimens, confirming that 14 of the DEGs had differential expression.

However, due to the unique properties of placental tissue, investigations are restricted to samples obtained at delivery. Both the sample tissues from the aforementioned two datasets and our subsequent validation tissues were obtained at delivery. Thus, it remains uncertain at which stage the changes in DEGs occur. If DEGs alterations occur during the early pregnancy of GDM patients, before the onset of hyperglycemia, these DEGs may represent critical molecules that trigger the disease. Conversely, if DEGs changes take place in the middle to late stages of pregnancy in GDM patients, characterized by high glucose and insulin resistance, such changes may arise as a result of altered bodily environment or compensatory mechanisms. To enhance our comprehension of the involvement of DEGs in the onset and progression of GDM and to effectively translate this knowledge into clinical applications, further comprehensive mechanistic investigations are imperative. This is particularly crucial considering that treatment strategies can significantly differ depending

Table 2. Summary of the DEGs expressions and correlation analysis of DEGs and blood glucose levels.

	Placenta specimens GDM v. Normal	HTR8/SVneo cells HG v. Normal	Correlation between DEGs expression and blood glucose level				
			First trimester	OGTT-0 h	OGTT-1 h	OGTT-2 h	Third trimester
HLA-E	↓	↓	-	*	*	-	-
CST7	NS	NE	-	-	-	-	-
SLAMF	↑	NS	-	-	-	-	-
MEDAG	NS	NS	-	-	*	-	-
MYH11	↓	NE	-	*	-	-	-
ALDH1A2	↑	NE	-	-	*	*	-
HLA-DRB5	↓	NS	-	*	*	*	-
BCL6	NS	↓	-	-	-	-	-
ITGAX	↓	NE	-	-	-	-	-
GZMB	↓	NE	-	-	-	-	-
NAIP	↓	↓	-	*	-	-	-
NDUFA6	NS	NS	-	-	-	-	-
SERPINA3	NS	↑	-	-	-	-	-
TMEM74B	↓	NS	-	*	*	*	-
RANBP3L	↓	NE	-	-	-	-	-
PAEP	↓	↓	-	-	*	-	-
CHI3L2	↑	NE	-	-	-	-	-
MUC16	NS	↓	-	*	-	*	-
WT-1	↓	↓	-	-	*	*	-
CEP170	↓	↓	-	-	-	-	-

Up arrow marked in red: the expressions of DEGs were increased in GDM placenta/high-glucose-treated HTR8/SVneo cells. Down arrow marked in blue: the expressions of DEGs were decreased in GDM placenta/high-glucose-treated HTR8/SVneo cells.
DEG: differentially expressed genes; GDM: gestational diabetes mellitus; OGTT: oral glucose tolerance test; NS: no significant difference; HE: SVneo cells genes may not express in HTR8/; -: no correlation; Red *: positive correlation; Blue *: negative correlation.

on the underlying etiology of the disease. For instance, in a study by Bai *et al.*, it was reported that the expression and secretion of ANGPTL8 were found to be up-regulated in the placenta of women diagnosed with GDM. Through further analysis, they identified that silencing of ANGPTL8 alleviated insulin resistance in trophoblast cells, providing a novel insight for diagnosis and treatment of GDM in clinic.[29] Liu *et al.* found that CSE O-GlcNAc and H2S production increased in the placenta of women with pre-eclampsia, but after further investigation into the underlying mechanism, they discovered that the increased CSE O-GlcNAc and H2S production were actually a compensatory response of the placenta,[13] and if we misinterpret this response as the cause of the disease and intervene, it may worsen the condition.

Therefore, to investigate the origin of differential gene expressions, we examined the expressions of all DEGs in HTR8/SVneo cells exposed to high glucose. We observed that the down-regulation of *i*, NAIP, PAEP, WT-1, and CEP170 expressions were consistent with the trend observed in GDM, indicating that these changes may be attributed to the high environment, which can either cause damage or trigger compensatory responses. The gene variants of leukocyte antigen (HLA) family have been linked to susceptibility to type 1 diabetes mellitus,[30] and in the context of GDM, there is a correlation between the presence of the condition and an elevated presence of anti-HLA-class II antibodies in the maternal circulation.[31] Neuronal apoptosis inhibitory protein (NAIP) is a constituent of the NLRC4 inflammasome,[32,33] and changes in NAIP expression in GDM may result from high glucose exposure, which could cause placental dysfunction. Progestagen-associated endometrial protein (PAEP), also known as glycodelin, is considered a paracrine regulator during early pregnancy.[34] In addition, Centrosomal protein 170 kDa (CEP170) and Wilms Tumor-1 (WT-1) are associated with diabetic complications.[35,36] Additional investigations are warranted to elucidate the precise mechanisms underlying the role of these DEGs in GDM and diabetic complications.

The differential expression of SLAMF, HLA-DRB5, and TMEM74B in GDM placenta was not observed in high-glucose cell model. This finding suggests that these gene changes may not be caused by the high-glucose environment and may instead play a role in the occurrence and development of the disease. Signaling lymphocytic activation molecule family (SLAMF) can increase the sensitivity of T cells to IL-2 and up-regulate the expression of CD25, promoting Treg differentiation from naïve CD4(+) T cells.[37] Our study confirmed the up-regulation of SLAMF in GDM placenta, which theoretically leads to an increase in Treg differentiation. However, Shao *et al.* observed a higher Th17/Treg ratio in GDM patients compared to controls,[38] which seemingly contradicts our conclusion. The possible reason is that under normal circumstances, Th17/Treg balance is essential for maintaining a normal pregnancy.[39] However, in GDM, the placental immune microenvironment is impaired, leading to an imbalance of Th17/Treg and a compensatory increase in placental SLAMF expression, which may potentially contribute to the differentiation of naïve CD4(+) T cells into regulatory Tregs. Nevertheless, additional investigations are necessary to confirm and validate this hypothesis.

The expression of *i*, *i*, ITGAX, GZMB, RANBP3L, and CHI3L2 genes were found to be altered in GDM placenta;

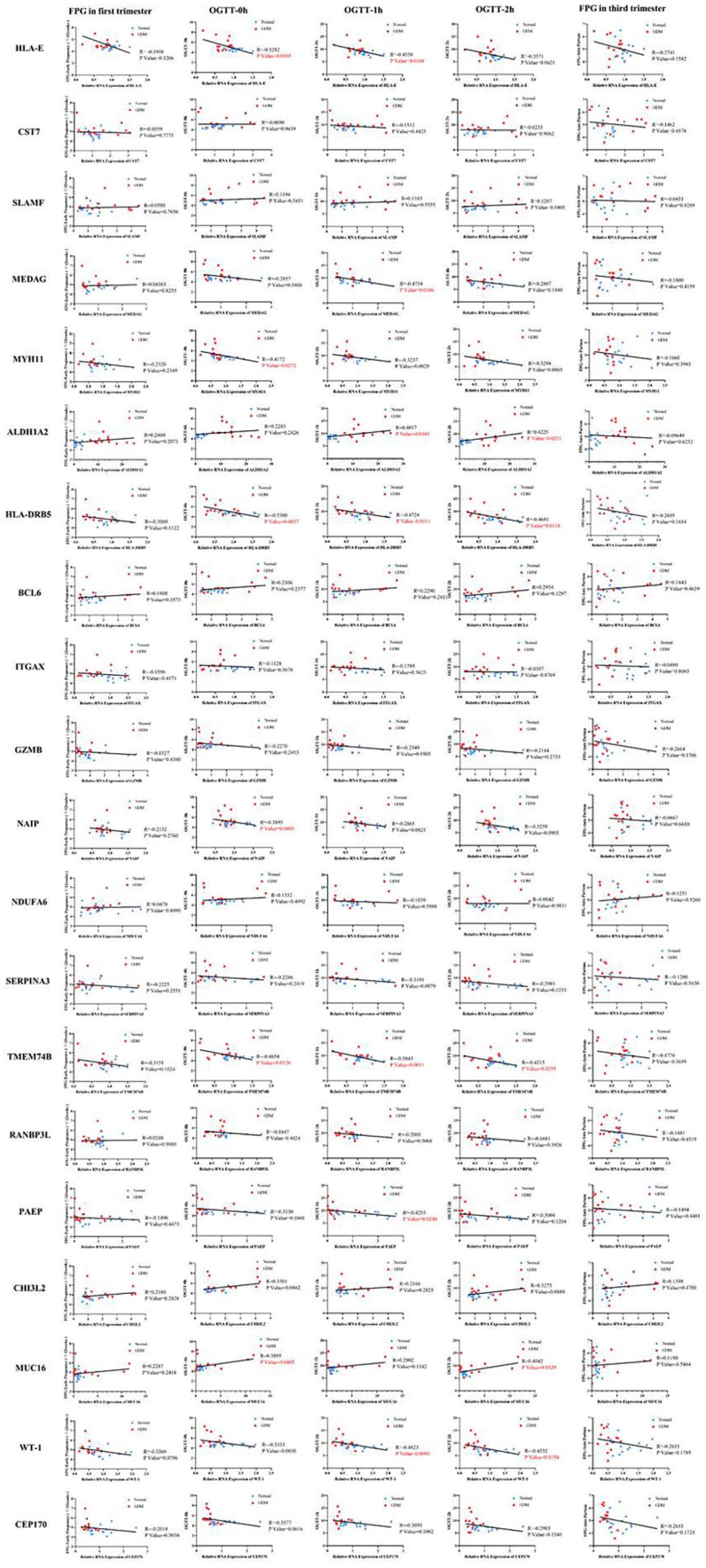

Figure 5. Correlation analysis of DEGs with maternal blood glucose levels. Red dots: GDM pregnant women; blue dots: normal pregnant women. Indicators with $P < 0.05$ are marked in red font.

however, these genes may not be expressed in HTR8/SVneo cells. This phenomenon can be attributed to the fact that the placenta contains not only trophoblast cells but also cells of both maternal and fetal origin, such as decidual cells, immune cells, umbilical vein endothelial cells, maternal endothelial cells, mesenchymal stromal cells, and so on.[40] These genes may not be expressed in trophoblast cells but in other cells, which are also important components of the placenta. Thus, the analysis of placental gene expression should not be limited to trophoblast cells because the placenta is a highly complex structure that requires comprehensive analysis and interpretation.

Interestingly, while there were no significant differences in the expression levels of SERPINA3 and BCL6 between GDM and normal placentas, their expression patterns were found to be altered (SERPINA3 was up-regulated while BCL6 was down-regulated) in HTR8/SVneo cells exposed to high glucose. Serine proteinase inhibitor A3 (SERPINA3) has been implicated in the pathogenesis of many diseases, including obesity, diabetes, cardiovascular diseases, and kidney disorders.[41] A previous study has also identified SERPINA3 as an important marker for type 2 diabetes mellitus through genome-wide gene expression differences analysis.[42] However, to date, there have been no studies on the relationship between SERPINA3 and GDM, making it a subject of great research value. B cell lymphoma 6 (BCL6) is considered to be involved in the pathogenesis of both type 1 and type 2 diabetes mellitus[43,44] and also has high research value in GDM.

In our study involving 28 pregnant women, we observed that pre-pregnancy BMI was significantly higher in women with GDM than in normal women, which is consistent with previous findings.[45–47] Notably, we observed differences in FPG levels between women with GDM and normal pregnant women as early as the first trimester, highlighting the importance of early testing of FPG. If the FPG is elevated in early pregnancy, it is advisable to start managing it through diet or exercise to avoid the need for intervention only after the diagnosis of GDM is made by OGTT testing in mid-pregnancy. In addition, although previous research has suggested that macrosomia is a common complication of GDM,[48,49] our study did not find significant differences in birth weight among the newborns. This lack of significant findings could be attributed to the limited sample size of our study, which may have affected the statistical power and ability to detect true differences.

Li *et al*. identified a positive correlation between placental IL-15 and blood glucose levels at three time points in the OGTT, indicating that IL-15 could serve as a biomarker of GDM and hold translational medicine value.[50] To investigate the relationship between DEGs and blood glucose levels, we conducted a correlation analysis between the expression levels of all DEGs and blood glucose levels. Our analysis revealed that the expression levels of ALDH1A2 and MUC16 exhibited a positive correlation with blood glucose levels, whereas the expression levels of HLA-E, MEDAG, MYH11, HLA-DRB5, NAIP, TMEM74B, PAEP, and WT-1 were negatively correlated with blood glucose levels. These findings suggest that these DEGs may be involved in the regulation of blood glucose homeostasis. These correlations were evident during the OGTT but not in the first or third trimester, implying that the expression changes of these DEGs may be more closely linked to impaired glucose tolerance rather than impaired fasting glucose. If the expression differences of these genes can be validated in peripheral blood in early pregnancy, it would have a tremendous predictive value for GDM. Further validation with large-scale samples is warranted.

Our study has several limitations that should be acknowledged. First, the validation of DEGs was performed using RT-qPCR instead of western blot, which may have led to less accurate results. Second, the sample size was relatively small, which may have resulted in errors in the results. Third, although some DEGs were found to be correlated with blood glucose levels, further studies are required to elucidate the underlying mechanisms. Furthermore, it should be noted that the process of selecting DEGs through the intersection of two datasets may inadvertently overlook certain important genes that hold potential significance in the development of GDM. Therefore, it is crucial to consider the inclusion of these genes in future investigations.

Conclusions

In summary, our study employed a multifaceted approach to identify 20 potential key genes in placental tissues of GDM and validate them in placental specimens. To explore the causality, we verified changes in the expression of these genes in high-glucose cell model. In addition, we associated these DEGs with blood glucose levels, providing a possibility for clinical translational medicine. Our research findings offer novel insights into the study of placenta in GDM. The identified DEGs hold significant potential for advancing the prevention and treatment strategies for GDM, ultimately benefiting the birth outcome and long-term prognosis of fetuses in GDM patients.

AUTHORS' CONTRIBUTIONS

Conceptualization, YJ and YD; methodology, YJ; software, YJ and RS; validation, YJ, LW, HZ, YC, and PG; formal analysis, XZ; investigation, SZ, CF, LF, and SW; resources, YJ, JZ, and RS; data curation, JY, LF, and SW; writing—original draft preparation, YJ and JZ; writing—review and editing, LW and HZ; visualization, YJ; supervision, PG, SZ, and XZ; project administration, WD; funding acquisition, LF. All authors have reviewed and consented to the publication of the manuscript in its final form.

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DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

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