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Identification of key ubiquitination-related genes in gestational diabetes mellitus: A bioinformatics-driven study

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Abstract

Background and Aims: Gestational diabetes mellitus (GDM) is characterized by glucose intolerance that occurs during pregnancy. This study aimed to identify key ubiquitination-related genes associated with GDM pathogenesis.

Methods: Microarray data from GSE154377 was analyzed to identify differentially expressed genes (DEGs) in GDM vs normal pregnancy samples. Weighted gene co-expression network analysis was performed on ubiquitination-related genes. Functional enrichment, protein-protein interaction network, and TF-mRNA-miRNA interaction network analyses were conducted on differentially expressed ubiquitination-related genes (DE-URGs).

Results: We identified 2337 DEGs and 65 DE-URGs in GDM. Functional enrichment analysis of the 65 DE-URGs revealed involvement in protein ubiquitination and ubiquitin-dependent catabolic processes. Protein-protein interaction network analysis identified 8 hub genes, including MAP1LC3C, USP26, USP6, UBE2U, USP2, USP43, UCHL1, and USP44. ROC curve analysis showed these hub genes have high diagnostic accuracy for GDM (AUC > 0.6). The TF-mRNA-miRNA interaction network suggested USP2 and UCHL1 may be key ubiquitination genes in GDM.

Conclusion: In conclusion, this study contributes to our understanding of the molecular landscape of GDM by uncovering key ubiquitination-related genes. These findings may serve as a foundation for further investigations, offering potential biomarkers and therapeutic targets for clinical applications in GDM management.

KEYWORDS

bioinformatics, gestational diabetes, microarray, network analysis, ubiquitination

1 | INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as glucose intolerance that begins or is first diagnosed during pregnancy.[1] The prevalence of GDM is increasing worldwide due to risk factors such as advanced maternal age and obesity.[2] GDM poses threats to both maternal and fetal health, and is associated with adverse pregnancy outcomes including pre-eclampsia, cesarean delivery, macrosomia, and perinatal mortality.[3] Moreover, a history of GDM predisposes mothers and offspring to developing type 2 diabetes, metabolic syndrome, and

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cardiovascular disease later in life.[4] The pathophysiology of GDM is not fully understood but likely involves both insulin resistance and impaired insulin secretion.[5]

Pregnancy hormones including placental lactogen, prolactin, and cortisol, as well as adipokines from adipose tissue can antagonize insulin action and contribute to insulin resistance.[6] Protein ubiquitination is a dynamic posttranslational modification that regulates diverse cellular processes.[7] Ubiquitin chains linked through different lysine residues create distinct topological signaling platforms that determine protein fate and function.[8] Previous study demonstrated that the ubiquitin-proteasome system (UPS) plays crucial roles in maintaining cellular homeostasis, and their dysregulation has been implicated in various metabolic diseases, including obesity, insulin resistance, and diabetes mellitus.[9–11] Aye ILMH et al. identified adiponectin ubiquitination as a critical mechanism through which obesity diminishes adiponectin secretion during pregnancy to influencing maternal insulin resistance and fetal growth in pregnancy.[12] A transcriptomic profiling study of trophoblast isolated from GDM patients showed that 8 ubiquitin-conjugating enzymes (UBE) splice variants were associated with increased maternal fasting plasma glucose.[13] Another study showed that the genetic variants of UBE2E2 were associated with GDM.[14] However, the connection between ubiquitination and gestational diabetes remains poorly studied.

In this study, we aimed to identify key ubiquitination-related genes associated with the pathogenesis of GDM using an integrated bioinformatics approach. Microarray data were analyzed to detect differentially expressed genes (DEGs), followed by weighted gene co-expression network analysis (WGCNA) to identify significant modules and hub genes related to GDM. Enrichment analysis revealed involvement of ubiquitination pathways in GDM. Upstream regulation analysis also provided insights into potential mechanisms underlying ubiquitination mediated GDM. These findings may help elucidate the complex ubiquitination mediated molecular events underlying GDM and reveal novel biomarkers or therapeutic targets for GDM treatment.

2 | MATERIALS AND METHODS

2.1 | Microarray data processing and DEG screening

The microarray data set GSE154377 was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/).[15] GEO belong to public databases. Users can download relevant data for free for research and publish relevant articles. Our study is based on open source data, so there are no ethical issues and other conflicts of interest. And according to the statements of the database source article, the Ethics Committee had authorized construction of this database, and informed consents of all patients were obtained. This data set analyzed the cell-free RNA content of 44 normal pregnant patients, 33 GDM patients, 40 pre-eclampsia or gestational hypertension patients, 10 chronic hypertension patients, and 7 nonpregnant patients. It covers samples from early, middle, and late pregnancy, as well as at delivery, providing comprehensive time-series data, which aligns with our objective of investigating GDM molecular mechanisms and allowing us to identify potential biomarkers and pathways involved in GDM pathogenesis. In the present study, we only adopted normal pregnant and GDM samples to analyze.

2.2 | Data processing and differential gene screening

The Count data was converted to TPM data using R language as the previous study mentioned.[16] Briefly, we used the following formula: TPM = (read_counts * 10^6)/(gene_length*sum (read_counts/gene_length)). Gene symbols were converted to standard official gene symbols, taking the average values when multiple ENSG IDs mapped to the same symbol. DEGs were identified by DEseq. 2 with thresholds of $|\log2FC| > 1$ and adjusted p-value < 0.05 consisted with the previous mentioned study.

2.3 | Acquisition of ubiquitination-related genes

A total of 1342 ubiquitination-related genes were obtained from the iUUCD 2.0 database (http://iuucd.biocuckoo.org/index.php).[17]

2.4 | WGCNA analysis

The R package WGCNA was used to further process the 1342 discovered ubiquitination-related genes to construct a weighted gene co-expression network for the ubiquitination-related genes in GDM and normal samples.[18] The goodSamplesGenes function was used to screen the GDM and normal expression matrices and remove unqualified genes and samples. Next, the h-clust was used to cluster samples to remove outliers. We found that GSM4669966 and GSM4669901 were outliers and removed them from subsequent analysis.

Studies have shown that co-expression networks conform to scale-free networks,[19,20] that is, the logarithm log(k) of the degree of connection k of nodes appears negatively correlated with the logarithm log(P(k)) of the probability that nodes of degree k occur, with a correlation coefficient greater than 0.9. To ensure that the network was scale-free, we chose the optimal soft threshold of 6. The next step was to transform the expression matrix into an adjacency matrix, and then transform the adjacency matrix into a topological matrix. Based on TOM, we used average linkage hierarchical clustering to cluster genes and set the minimum number of genes in each gene network module to 30 using the cutreeDynamic algorithm. After determining the gene modules with the dynamic tree cut, we calculated the eigengene value for each module at a time, then clustered the

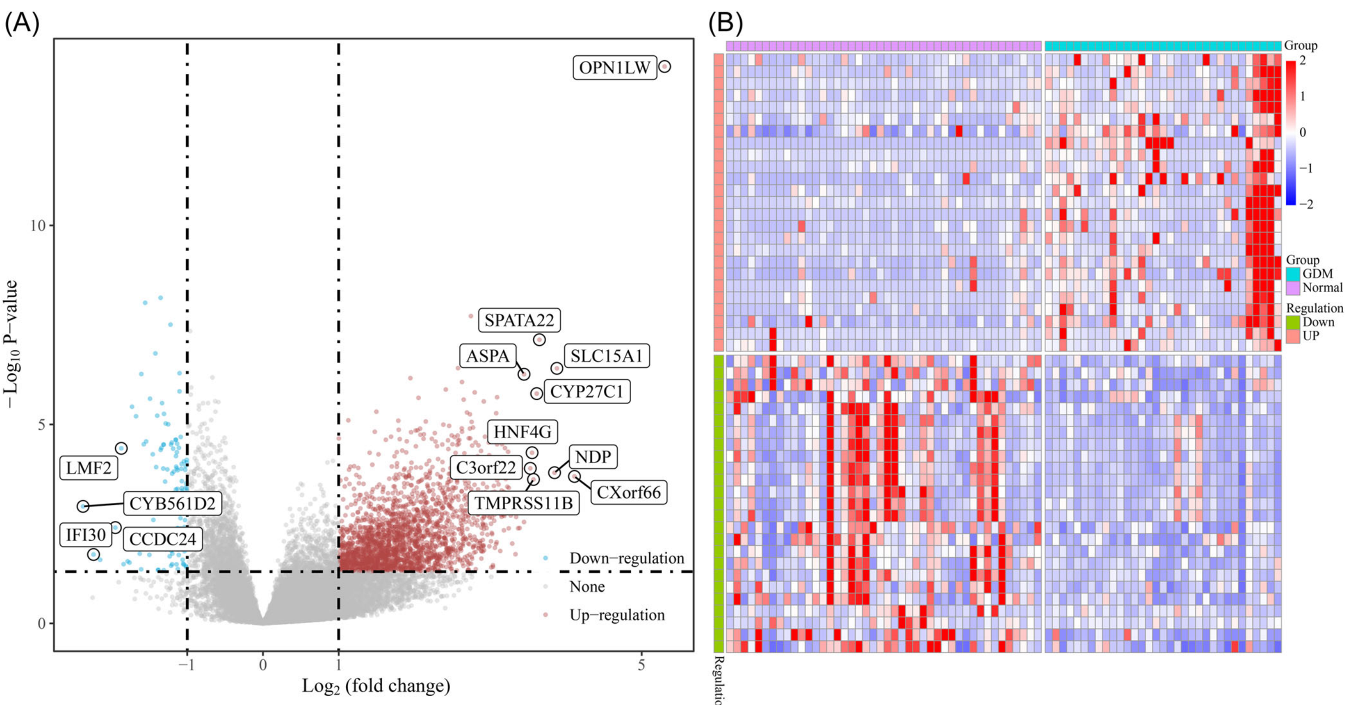

FIGURE 1 Volcano plot of differentially expressed genes. (A) Blue represents downregulated genes, red represents upregulated genes, and gray represents non-significantly differentially expressed genes. (B) Heatmap of the top 50 differentially expressed genes. Red represents genes with higher expression values and blue represents genes with lower expression values.

modules to merge modules with close distances into new modules, setting the height at 0.25. In total 5 modules were obtained.

We then calculated the Pearson correlation coefficients between these 5 modules and GDM and normal samples. We found that the yellow module was significantly negatively correlated with GDM and the turquoise module was significantly positively correlated with GDM. These two modules were the top two modules that were significantly correlated with clinical features. The yellow and turquoise modules showed significant positive correlations between the MM and GS of the target genes. Therefore, the yellow and turquoise modules were considered to be key modules. Next we took the intersection of genes in the key modules and differential genes, whereby intersection genes were considered differentially expressed ubiquitination-related genes (DE-URGs). The "ggplot2" package was used to display the expression of DE-URGs in heatmaps.

2.5 | GO and KEGG functional enrichment analysis of DE-URGs

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis for DE-URGs was performed using the R package clusterProfiler to determine potential biological functions.[21] Functional enrichment analysis was performed using the R package clusterProfiler. Enrichment results were sorted according to adjusted P values. The bar plot shows the first 10 results.

2.6 | Construction of DE-URG PPI interaction network

The online database STRING (https://string-db.org/) was used to construct a protein-protein interaction network for DE-URGs.[22] In this process, "Homo sapiens" was selected as the biological species, the network type was set to "full STRING network", the required score was set to "low confidence", and the strictness of FDR (False Discovery Rate) was set to 0.05. The network was visualized using Cytoscape.[23] Hub genes were defined as the intersection of the top 10 nodes ranked by maximal clique centrality (MCC), maximum neighborhood component (MNC), degree, edge percolated component (EPC), and closeness centrality. ROC curve analysis was done to evaluate the diagnostic performance of hub genes.

2.7 | Construction of TF-gene-miRNA interaction network

The RNA Interactome Database (RNAInter) collects over 40 million entries of various types of RNA interactions from the literature and more than 30 RNA-related databases. It combines annotation information such as RNA editing, localization, modification, target areas, structure, and homologous interactions. We obtained transcription factors regulating hub genes (score > 0.1) from the RNAInter database (http://www.rna-society.org/rnainter/). In addition, we also

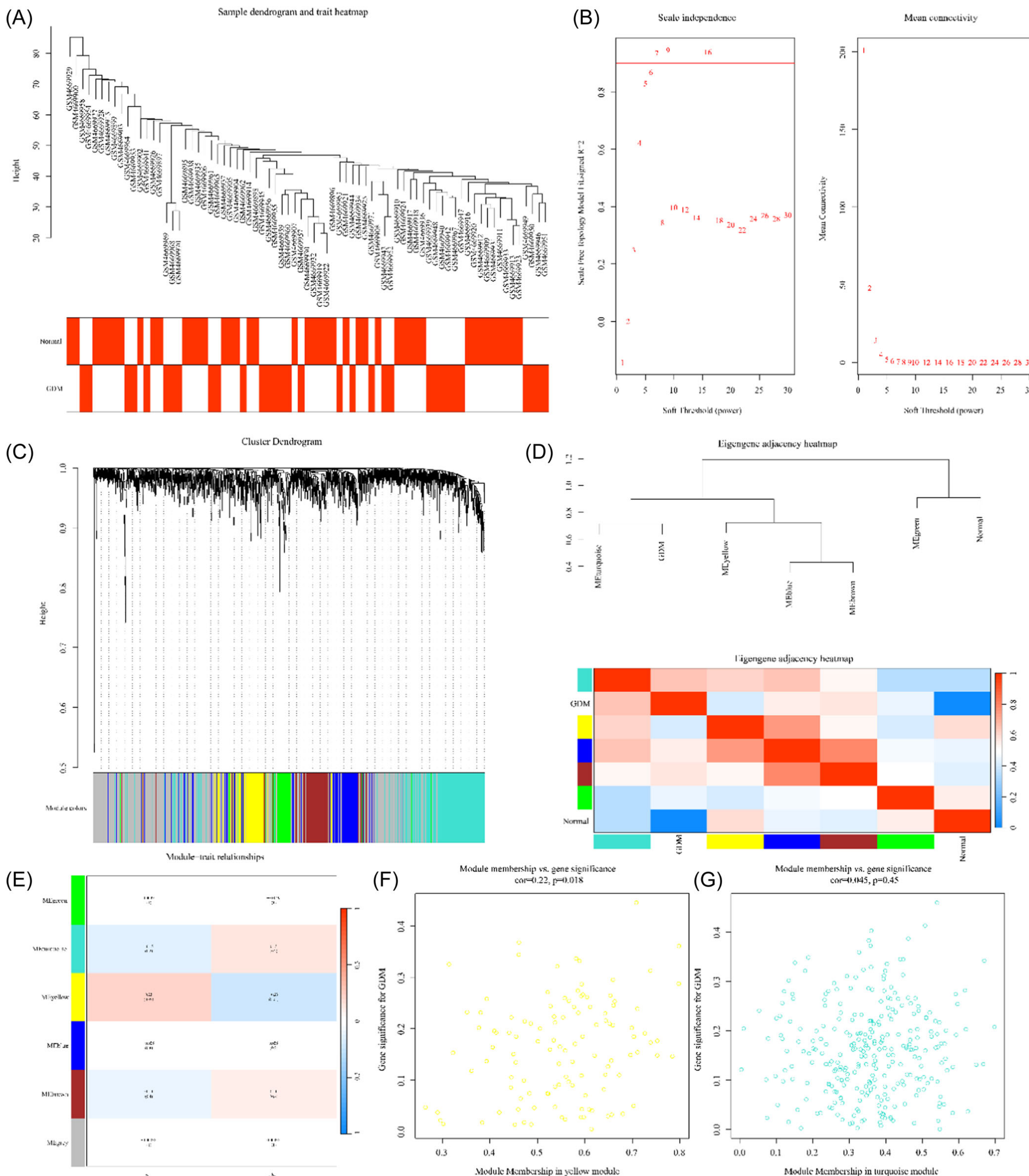

FIGURE 2 WGCNA analysis of ubiquitination-related genes. (A) Sample clustering tree to check outliers. (B) Mean connectivity and scale-free fit index for various soft-thresholding powers. (C) Cluster dendrogram based on topological overlap matrix. (D-E) Heatmaps of module eigengene correlation with GDM patients or controls. (F-G) Scatterplots of module membership vs gene significance for yellow and turquoise modules.

obtained hub gene-miRNA interaction data (score > 0.2) from RNAInter.[24] Finally, we used Cytoscape to visualize the TF-mRNA-miRNA interaction network.[23]

3 | RESULTS

3.1 | Identification of differentially expressed genes

This study used the GSE154377 data set downloaded from the GEO database, including 44 normal pregnant samples and 33 GDM samples. We identified 2337 DEGs (Supplementary Table 1) in GDM versus normal pregnancies using the R package DEseq. 2. Of these, 2192 were highly expressed in GDM and 145 were lowly expressed in GDM ($|\log2FC| > 1$, p value < 0.05) (Figure 1 A). The heat map shows the distribution of the top 50 DEGs in normal pregnancy and GDM (Figure 1B). Among them, HNF4G was reported to be associated with beta cell development and may be responsible for a decrease in beta cell mass.[25] The polymorphism at the IFI30 locus was associated with the risk of hyperglycemia/diabetes in severely obese individuals.[26] However, the potential function of these genes underlying GDM were poorly understood and need more studies.

3.2 | WGCNA analysis

The WGCNA package in R was used to further process the 1342 discovered ubiquitination-related genes. In constructing the sample tree, GSM4669966 and GSM4669901 were abnormal samples, so they were removed in subsequent analysis (Supplementary Figure S1, Figure 2A). A scale-free network of R2 > 0.9 was established with a soft threshold of 6 (Figure 2B). The gene set was divided into 5 modules with a minimum module size of 30 genes (Figure 2C). The correlation of each module with GDM was determined (Figures 2D and 2E). The yellow and turquoise modules were the most negatively and positively related modules to GDM, respectively. These two modules were the first two modules that were significantly correlated with clinical features. The yellow and turquoise modules showed significant positive correlations between the MM and GS of the target genes (Figures 2F and 2G). Therefore, the yellow and turquoise modules were considered to be key modules. The detailed biological significance of the yellow and turquoise modules were represented in supplementary materials Figure S2 and Figure S3.

3.3 | Identification of differentially expressed ubiquitination-related genes (DE-URGs)

The 2337 differential genes identified by DEseq. 2 were crossed with the genes in key modules to determine DE-URGs. A total of 65 differentially expressed ubiquitination-related genes were identified, including 64 positively correlated genes and 1 negatively correlated gene (Figure 3).

3.4 | Functional enrichment analysis of differentially expressed ubiquitination-related genes

The R package clusterProfiler was used to perform GO and KEGG enrichment analysis on DE-URGs to determine their potential physiological functions. Bar plots show the top 10 results. The most significant enrichment items were protein autoubiquitination, protein ubiquitination, protein deubiquitination, protein catabolic process of proteolysis, modification-dependent macromolecule catabolic process, ubiquitin-dependent protein catabolic process, protein polyubiquitination (Biological Process), cullin-RING ubiquitin ligase complex (Cellular Component), deubiquitinating enzyme activity, ubiquitin-like protein conjugating enzyme activity, ubiquitin-protein transferase activity, ubiquitin-like protein transferase activity, ubiquitin-specific peptidase activity, zinc ion binding, thiol-dependent peptidase activity, thiol-type deubiquitinase activity, ubiquitin-protein transferase activity, thiol-type endopeptidase activity (Molecular Function) (Figure 4).

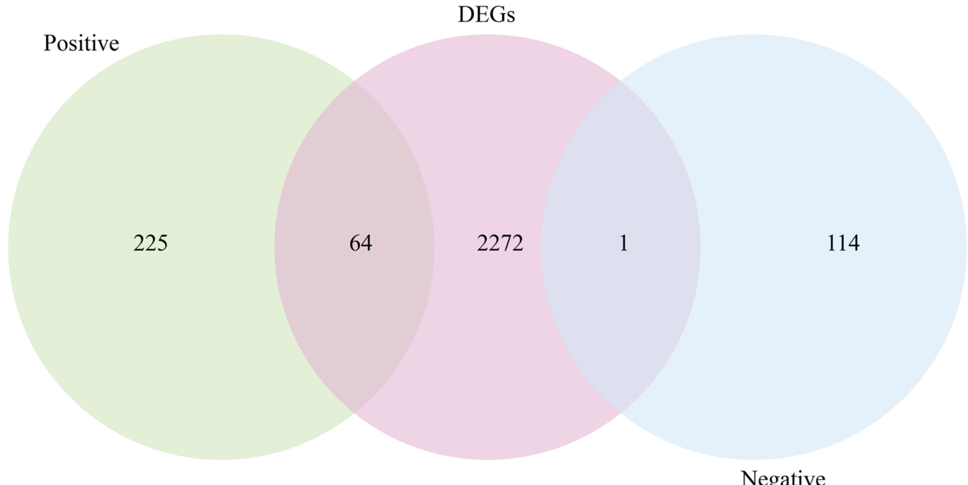

FIGURE 3 Intersection of differentially expressed genes with positively and negatively correlated module genes.

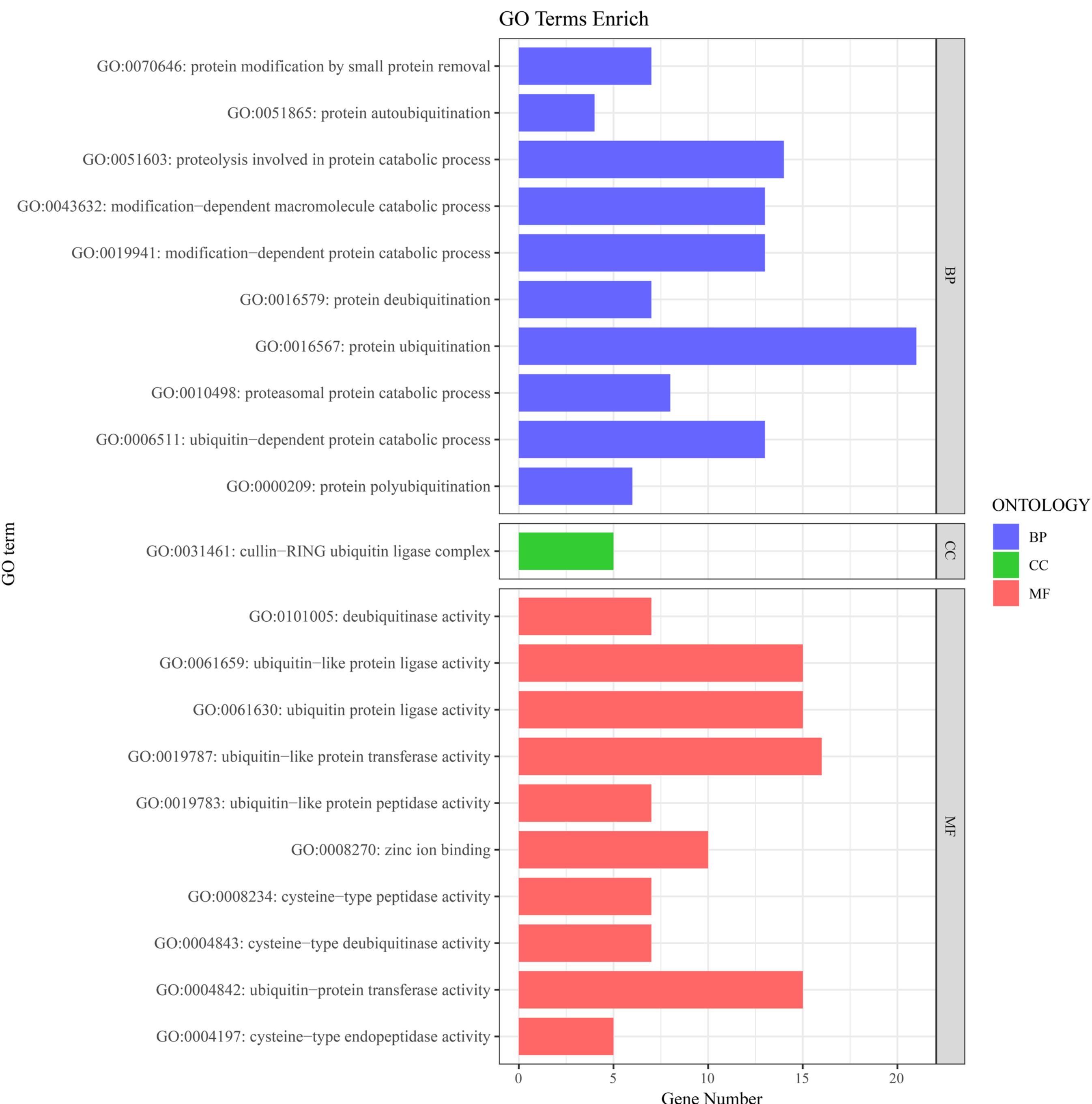

FIGURE 4 Functional enrichment analysis of DE-URGs.

3.5 | Construction of PPI interaction network for differentially expressed ubiquitination genes

To demonstrate the interactions between DE-URGs, we created a protein-protein interaction network (Figure 5). We obtained a PPI network with 68 nodes and 324 edges (Figure 5A). Table 1 shows the top 10 hub genes obtained by five algorithms (MCC, MNC, Degree, EPC, Closeness). Intersecting genes from the five algorithms were taken as hub genes. They were MAP1LC3C, USP26, USP6, UBE2U, USP2, USP43, UCHL1, USP44. Among them, these eight genes were all positively correlated genes.

We explored the diagnostic ability of these 8 genes in different patients and plotted the ROC curves. The results showed that the AUC of MAP1LC3C, USP26, USP6, UBE2U, USP2, USP43, UCHL1, USP44 were 0.3843, 0.4105, 0.6336, 0.3912, 0.6102, 0.6894, 0.6481, 0.6577, respectively (Figure 5B). The results indicate that the 5 genes USP6, USP2, USP43, UCHL1, USP44 have relatively high diagnostic accuracy as new biomarkers. And we compared the AUC

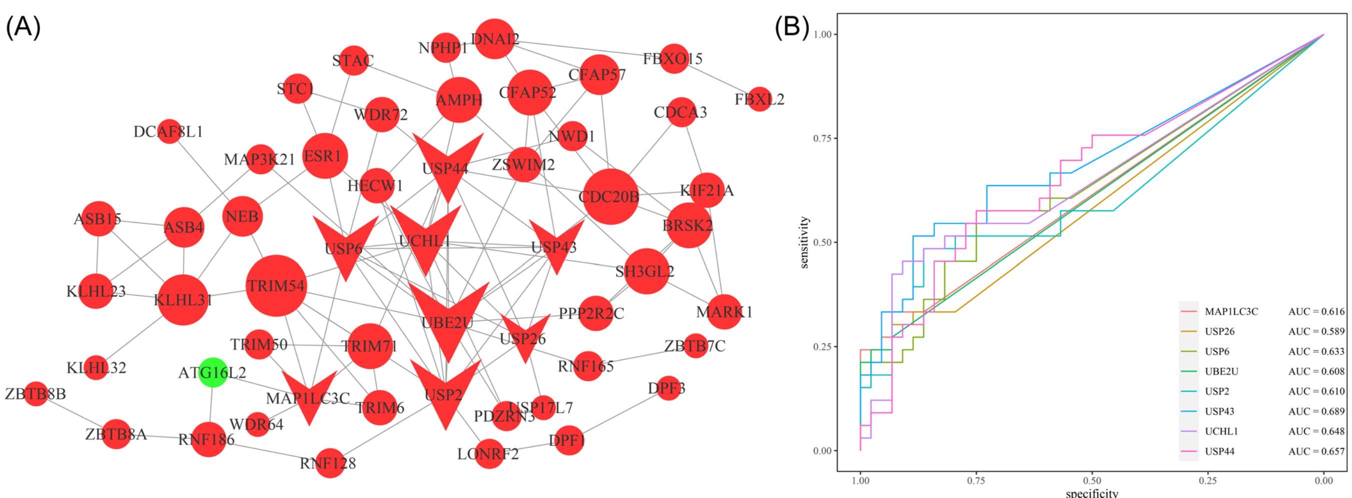

FIGURE 5 Construction of PPI network and identification of hub genes for DE-URGs. (A) PPI network of 65 DE-URGs. Green represents negatively correlated genes, red represents positively correlated genes. V nodes are hub genes; circles are others. (B) ROC curves judging GDM diagnostic performance of 8 genes.

TABLE 1 Top ten hub genes obtained by five algorithms of Cytohubba.

MCC	MNC	Degree	EPC	Closeness
MAP1LC3C	CFAP52	MAP1LC3C	MAP1LC3C	MAP1LC3C
USP26	MAP1LC3C	USP26	USP26	USP26
USP6	USP26	USP6	USP6	USP6
UBE2U	USP6	UBE2U	UBE2U	UBE2U
TRIM71	UBE2U	CDC20B	CDC20B	CDC20B
USP2	TRIM71	USP2	USP2	USP2
USP43	USP2	USP43	USP43	USP43
UCHL1	USP43	UCHL1	UCHL1	UCHL1
TRIM54	UCHL1	TRIM54	TRIM54	TRIM54
USP44	USP44	USP44	USP44	USP44

of several existing GDM biomarkers in this data set (showed in Figure. S4), the results showed that our 5 hub genes had the no-inferior predictive value.

3.6 | Construction of TF-mRNA-miRNA interaction network

TF-genes-miRNA interactions were collected through network analysis. Screening for interacting miRNAs and TFs of Hub genes (MAP1LC3C, USP26, USP6, UBE2U, USP2, USP43, UCHL1, USP44) (Figure 6). MAP1LC3C is regulated by 21 miRNAs, USP26 is regulated by 17 miRNAs, USP6 is regulated by 134 miRNAs, UBE2U is regulated by 12 miRNAs, USP2 is regulated by 91 miRNAs, USP43 is regulated by 25 miRNAs, UCHL1 is regulated by 84 miRNAs, USP44 is regulated by 76 miRNAs (Supplemental table 2). In the TF regulation network, USP26 is regulated by 1 transcription factor, USP6 is regulated by 1 transcription factor, UBE2U is regulated by 1 transcription factor, USP2 is regulated by 174 transcription factors, UCHL1 is regulated by 78 transcription factors, and USP44 is regulated by 2 transcription factors, indicating a high degree of interaction between TFs and hub genes.

4 | DISCUSSION

The ubiquitination signaling pathway is a ubiquitous protein modification pathway that regulates protein stability, function, interactions and localization, thereby affecting various cellular processes.[27] The role of the ubiquitination signaling pathway in gestational diabetes mellitus (GDM) has been partially studied. Studies have shown that the ubiquitination signaling pathway can regulate the ubiquitination and degradation of insulin receptor and insulin receptor substrate, thereby affecting insulin signal transduction.[28,29] Additionally, the ubiquitination signaling pathway can modulate the ubiquitination and degradation of glycogen synthase and glycogen phosphorylase, thus influencing hepatic glucose metabolism.[30,31] Furthermore, the ubiquitination signaling pathway can regulate autophagy and apoptosis of pancreatic β cells, thereby impacting insulin secretion.[32] Therefore, the ubiquitination signaling pathway may affect the pathogenesis and progression of GDM by modulating insulin signaling, glucose metabolism, and pancreatic β cell function. However, which ubiquitination related proteins underlying GDM was still poorly understood.

Bioinformatics analysis provides a comprehensive, data-driven understanding of the molecular, genetic, and environmental factors underlying disease development, and to support the development of more effective diagnostic tools, therapeutic interventions, and public health strategies.[33–35] In this study, we used bioinformatics methods

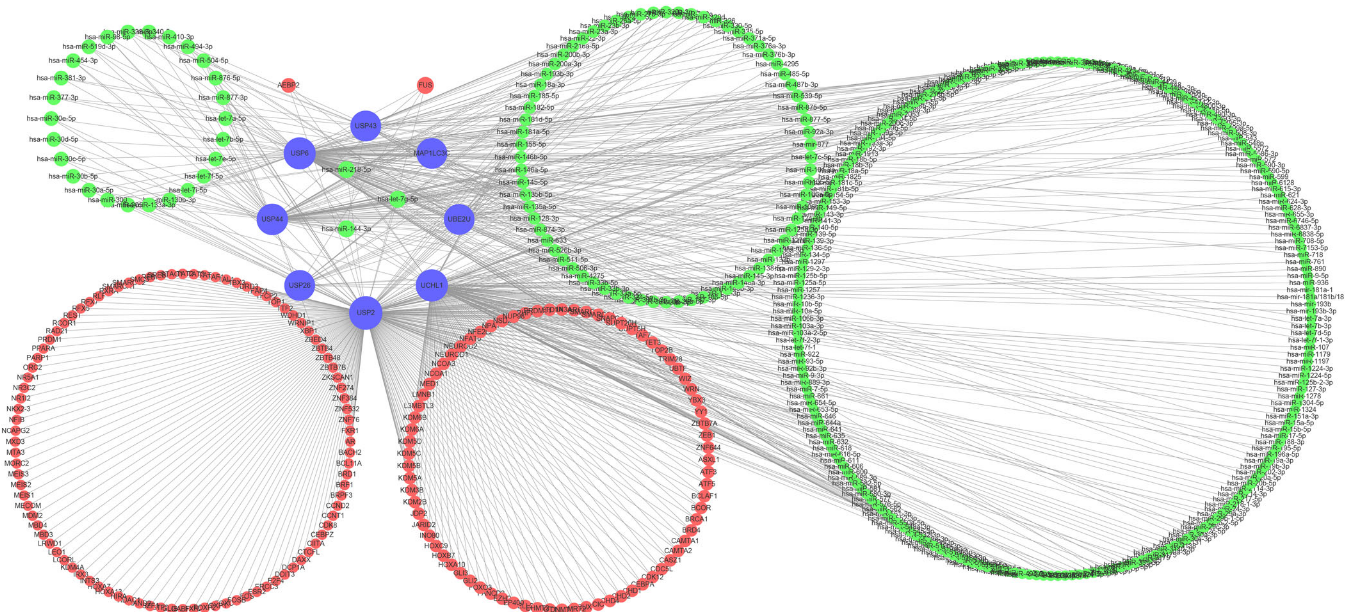

FIGURE 6 TF-genes-miRNA interaction network. Blue nodes represent hub genes, red nodes represent transcription factors, and green nodes represent miRNAs.

to screen for ubiquitination-related genes associated with gestational diabetes mellitus from the GSE154377 data set, and performed functional enrichment analysis and network analysis on them, revealing the role of ubiquitination in the pathogenesis of gestational diabetes mellitus. We were crossed with the genes found 65 ubiquitination-related genes differentially expressed in gestational diabetes mellitus and normal pregnancy samples, of which 64 were upregulated and 1 was downregulated. These genes were mainly involved in ubiquitination, protein degradation, cell cycle and apoptosis, and other biological processes, as well as ubiquitination signaling pathway, p53 signaling pathway, FoxO signaling pathway, and other signaling pathways, which had been shown to play an important role in the development of diabetes.[36,37] Such as ubiquitination pathway, it was reported to relate to insulin resistance in GDM.[29] P53 signaling pathway was reported to involve in the deterioration of GDM via activating the JAK/STAT signaling pathway.[38] These results suggest that ubiquitination may affect the occurrence and development of gestational diabetes mellitus by regulating protein homeostasis, cell fate and signal transduction.

The overlap of 2337 DEGs with ubiquitination-related genes to identify 65 differentially expressed ubiquitination-related genes (DE-URGs) provides strong biological plausibility for the involvement of ubiquitination pathways in the pathogenesis of GDM. The previous studies that have only focused on a few ubiquitination-related genes in GDM. For example, a transcriptomic profiling study of trophoblasts from GDM patients reported associations between 8 ubiquitin-conjugating enzyme splice variants and maternal fasting plasma glucose.[13] Another study found genetic variants of the ubiquitin-conjugating enzyme UBE2E2 to be associated with GDM.[14] The identification of 65 DE-URGs represents a novel and comprehensive discovery that advances our understanding of the molecular underpinnings of GDM. These results lay the groundwork for future studies to elucidate the specific roles of ubiquitination-related genes and their regulatory networks in the pathophysiology of GDM.

We further screened out 8 core genes from the ubiquitination-related genes, namely MAP1LC3C, USP26, USP6, UBE2U, USP2, USP43, UCHL1, USP44, and evaluated their diagnostic value for gestational diabetes mellitus. We found that, except for MAP1LC3C and UBE2U, the area under the ROC curve (AUC) of the other 6 genes were all greater than 0.6, indicating that they have relatively high diagnostic accuracy, which is comparable or even superior to some existing GDM biomarkers.[39,40] Combining these markers with existing screening markers to construct models may improve GDM diagnosis.

Among them, USP43 had the highest AUC, reaching 0.6894, indicating that USP43 may be an important cell-free DNA biomarker for gestational diabetes mellitus. This suggests that these hub genes could serve as promising cell-free DNA biomarkers for GDM screening and diagnosis. USP43 is a ubiquitin-specific protease that can remove ubiquitination modifications on proteins, thereby affecting their stability and function.[41] The role of USP43 in tumor was well studied, however, the relationship between USP43and GDM was still unknown.[42,43] And studies showed taht hypothalamic USP2 is likely necessary to maintain blood glucose levels at physiological concentrations.[10] Genetic ablation of UCHL1 was reported as a key molecule underlying type 2 diabetes, which leads to neuronal insulin resistance and T2D-related symptoms in Drosophila.[44] However, the potential functions of these hub genes underlying GDM need further research. There for, our results provide guidance for our next study in clinical validation and functional research.

The TF-mRNA-miRNA interaction network we constructed provides insights into the potential regulatory mechanisms of these hub

genes in GDM. The high number of regulatory interactions, particularly for USP2 and UCHL1, suggests complex regulation of these genes. This network analysis lays the groundwork for future experimental studies to validate these regulatory relationships and their functional consequences in GDM.

In conclusion, our study is the first to systematically analyze the role of ubiquitination-related genes in gestational diabetes mellitus, providing a new perspective for understanding the molecular mechanisms of gestational diabetes mellitus. Our study also provides new candidate targets for the diagnosis and treatment of gestational diabetes mellitus.

However, our study also has some limitations. First we employed only one data set to analyze, more GDM related data set should be coveraged to strengthen the validity of study outcomes. secondly, the ubiquitination-related genes were obtained from the iUUCD 2.0 database. It relies primarily on data extracted from peer-reviewed literature, which can be subject to publication bias, where studies with positive or novel findings are more likely to be published. This may lead to an over-representation of well-studied ubiquitin and ubiquitin-like conjugation pathways, while under-representing less understood or less frequently reported pathways. Thirdly, the function and regulation mechanism of the ubiquitination-related genes have not been verified in experiments, etc. Therefore, the results need to be further validated and expanded in larger cohorts and more experiments. Nevertheless, these findings have illuminated the trajectory to a certain degree for our future investigations.

AUTHOR CONTRIBUTIONS

Yuheng Dai: Data curation; Methodology; Writing—original draft; Conceptualization; Investigation; Validation; Software; Writing—review and editing; Formal analysis; Visualization. **Sha Lu**: Formal analysis; Methodology; Validation; Software; Data curation; Visualization; Writing—review and editing. **Wensheng Hu**: Project administration; Funding acquisition; Conceptualization; Methodology; Investigation; Supervision; Validation; Writing—review and editing; Resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available in the Gene Expression Omnibus repository, accession number GSE154377. The data that support the findings of this study are openly available in GEO database at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154377, reference number GSE154377.

TRANSPARENCY STATEMENT

The lead author Wensheng Hu affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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