Journal of Diabetes Research

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Research Article

Using Network Pharmacology to Explore the Mechanism of Panax notoginseng in the Treatment of Myocardial Fibrosis

Jingxue Han,[1,2] **Jingyi Hou**,[3] **Yu Liu**,[1,2] **Peng Liu**,[4] **Tingting Zhao**,[1] **and Xinwei Wang**[2]

[1]*Beijing Key Lab for Immune-Mediated Inflammatory Diseases, Institute of Clinical Medical Sciences, China-Japan Friendship Hospital, Beijing 100029, China*
[2]*Heilongjiang Academy of Chinese Medical Sciences, Harbin 150036, China*
[3]*School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing 100029, China*
[4]*Shunyi Hospital, Beijing Traditional Chinese Medicine Hospital, Beijing 101300, China*

Correspondence should be addressed to Tingting Zhao; ttfrfr@163.com and Xinwei Wang; chengguanghong77@sina.com

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Objective. The mechanism of Panax notoginseng in treating myocardial fibrosis (MF) was investigated using network pharmacology. *Methods*. Effective ingredients and potential targets of Panax notoginseng were screened in relevant databases to construct a compound-target network. Targets of MF were then screened to select common targets and construct a protein-protein interaction network. This was followed by Gene Ontology and pathway enrichment analyses. Molecular docking then verified the results of network analysis. *Results*. A total of 14 effective ingredients and 119 potential targets for MF were predicted. Quercetin, beta-sitosterol, and gossypetin were speculated to be the main active ingredients. The mechanism of action may be related to AGE-RAGE, proteoglycans, and IL-17 signaling pathways. Five key targets (IL6, ALB, AKT1, TNF, and VEGFA) may be involved in the treatment of MF using Panax notoginseng. *Conclusions*. This study embodies the complex network relationship of multicomponents, multitargets, and multipathways of Panax notoginseng in treating MF and provides a novel method for further research on this herb's mechanism.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, and it is estimated that CVD will result in more than 23 million deaths by 2030 [1]. Diabetes mellitus is widely known as a major risk factor for CVD. When glucose is not well-controlled in either type 1 or 2 diabetes, vascular and nerve damage can occur over time [2–4]. Damage to the heart vessels can lead to CVD. Myocardial fibrosis (MF) is a pathophysiologic process of many cardiovascular diseases [5, 6]. MF is the result of persistent and/or repeated damage and stress from various causes. These can include myocardial ischemia and hypoxia due to coronary atherosclerotic stenosis resulting from diabetes mellitus [7, 8]. Drugs such as angiotensin-converting enzyme inhibitors, beta-blockers, statins, and agents that target fibrosis have some beneficial effects. However, they cannot prevent the progression of MF and have certain side effects [9]. Thus, alternative therapies such as traditional Chinese medicine may be a treatment option for MF with fewer side effects and lower cost.

Panax notoginseng (Burk.) F.H. Chen (notoginseng) is an herb commonly used in Chinese medicine. Its traditional application is to promote blood circulation and dispel blood stasis. Records of Panax notoginseng date to the *Compendium of Materia Medica* (*Ben Cao Gang Mu*) compiled by Li Shizhen in the Ming dynasty. Modern research has revealed that the main components of Panax notoginseng include saponins, volatile oils, flavonoids, and polysaccharides. Its pharmacologic effects are mainly reflected in its

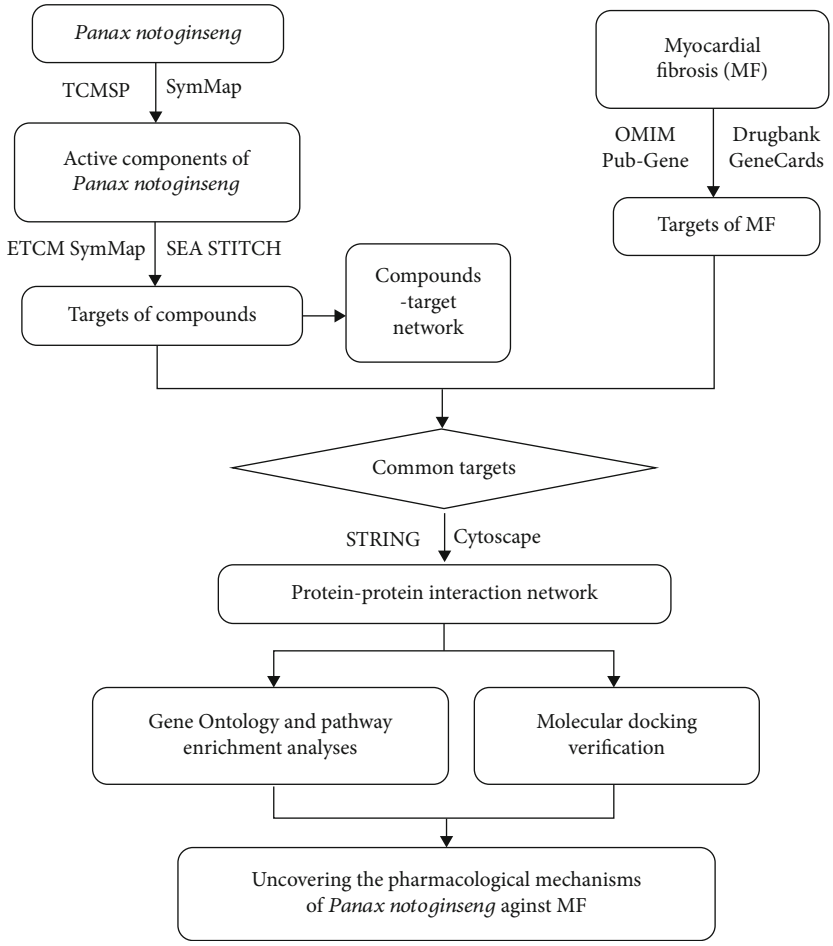


Figure 1: Flow diagram of the pharmacology-based study of Panax notoginseng used in treating MF.

actions on the circulatory and cerebrovascular systems. In murine experiments, Panax notoginseng has been found to have a therapeutic effect on MF [10–12]. However, its mechanism of action remains unclear.

Network pharmacology is a systematic research methodology that combines laboratory and clinical inquiries with data processing to guide drug discovery and development. It is an effective method for studying the complex relationship between Chinese herbal medicines and diseases [13]. This current study uses network pharmacology methods to elucidate the potential mechanism of Panax notoginseng in the treatment of MF and provides a basis for subsequent pharmacologic experimental studies (Figure 1).

2. Materials and Methods

2.1. Screening of Compound Components. The keywords "Panax notoginseng" were used to retrieve the compound components in the SymMap database (http://www.symmap.org) and in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database (https://tcmspw.com/tcmsp.php). The screening criteria were oral bioavailability (OB) ≥ 30% and drug-like (DL) ≥ 0.18.

2.2. Construction of the Component-Target Network. The targets of the compounds were searched through the Encyclopedia of Traditional Chinese Medicine (ETCM) database (http://www.nrc.ac.cn:9090/ETCM/index.php/Home/Index/index.html), the SymMap database, the Similarity Ensemble Approach (SEA) database (http://sea.bkslab.org), and the STITCH database (http://stitch.embl.de). The UniProt ID of the target was then searched through the Universal Protein Resource (UniProt) database (https://www.uniprot.org), with the species defined as "Homo sapiens." All gene names were assigned their official gene symbol. Then, targets that did not meet the screening criteria were eliminated. Next, the network mapping software Cytoscape 3.8.0 (http://www.cytoscape.org) was used to construct networks for the compounds and their targets. In the network, a node represents a target, gene, molecule, or protein, and the connections between nodes represent the interactions between the targets, genes, molecules, or proteins. The "degree" value of a node represents the number of connections between the nodes in the network; the larger the value, the more likely the target is to become the key target of compounds.

2.3. Acquisition of Disease Targets. The keyword "myocardial fibrosis" was searched in the Online Mendelian Inheritance in Man (OMIM) database (https://omim.org), GeneCards

database (https://www.genecards.org), Drugbank database (https://www.drugbank.ca), and Pub-Gene database (https://www.ncbi.nlm.nih.gov/pubmed) to obtain the disease targets.

2.4. Construction and Analysis of the Protein-Protein Interaction (PPI) Network. The potential targets of the retrieved compounds and disease targets were intersected, and the overlapping targets were selected and imported into the STRING database (https://string-db.org) to obtain the protein interaction relationship. The results were then imported into Cytoscape 3.8.0 software to construct and analyze the interaction network.

2.5. Screening of Core Clusters and Key Targets. Cytoscape plugin MCODE was applied for cluster analysis, and the filter conditions were set as degree cutoff: 2, *k*-core: 2 to select the core cluster with the closest relationship in the network. Then, the plugin CytoHubba was applied to analyze the PPI network and core cluster to obtain the network topology parameters. The targets shared by both the PPI network and core cluster with a high degree were selected as the key targets, which were retrieved in the DisGeNET database (http://www.disgenet.org/search) to obtain the protein class of key targets.

2.6. Gene Ontology and Pathway Enrichment Analyses. The Gene Ontology (GO) database (http://geneontology.org) includes various functions of genes including biologic process (BP), molecular function (MF), and cellular component (CC) and can be applied to the analysis of potential biologic molecular mechanisms. The KEGG database (https://www.kegg.jp) is used to identify biologic functions and candidate targets. In this study, ClusterProfiler (https://bioconductor.org/packages/release/clusterProfiler.html) in R package was applied to GO functional annotation and KEGG pathway analysis, and the enrichment analysis results were visualized.

2.7. Molecular Docking Verification. The Ligand Docking module in Schrödinger software was used to verify the reliability of the results, and the binding activity of the compound to the key targets was evaluated by the docking score. The structures of all the compounds were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and the three-dimensional structures of the key targets were downloaded from the Protein Database (PDB) (http://www.rcsb.org/pdb/home/home.do). The higher the absolute value of the docking score, the stronger the binding ability of small molecules to protease targets.

3. Results and Analysis

3.1. Compound Screening. Ten compounds were screened through the SymMap and TCMSP databases. The OB and DL values of notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, and ginsenoside Rb2 were smaller than the screening criteria and were deleted by the system. However, through searching the literature, we found that these compounds are related to myocardial fibrosis and diabetes and thus included the compounds [11, 14–16]. Therefore, a total of 14 compounds were eventually contained in the follow-up study (Table 1).

3.2. Target Prediction and Network Analysis of Compounds. Potential targets of compounds through ETCM, SymMap, SEA, and STITCH databases were searched, and 829 targets of Panax notoginseng were obtained after deleting duplicates.

Using Cytoscape 3.8.0, we constructed a network relationship among compounds and predicted targets (Figure 2). The resulting network included 451 nodes and 829 interaction edges. The degree values of compounds in the compound-target network were then obtained (Table 2). Quercetin has 238 potential targets, followed by beta-sitosterol with 121, gossypetin with 102, and stigmasterol with 96. These higher-degree compounds are likely to be involved in treatment of MF by Panax notoginseng.

3.3. Results of Disease Target Retrieval. With "myocardial fibrosis" as the keyword, a combined total of 601 myocardial fibrosis disease targets were found in the OMIM, Pub-Gene, Drugbank, and GeneCards databases after deleting duplicates.

3.4. Screening of Drug-Disease Targets. The intersections of potential targets of Panax notoginseng and disease targets resulted in 119 potential treatment targets for MF.

3.5. PPI Network of Panax notoginseng in the Treatment of MF and Key Target Analysis. The PPI network was mapped using common potential targets of Panax notoginseng and MF, consisting of 119 nodes and 2597 interaction edges (Figure 3(a)). The CytoHubba plug-in was used to analyze the PPI network to obtain core clusters (Figure 3(b)) and key targets (degree > 90). The following are the five targets with the largest degree value: interleukin 6 (IL6), albumin (ALB), AKT serine/threonine kinase 1 (AKT1), tumor necrosis factor (TNF), and vascular endothelial growth factor A (VEGFA), whose protein class involves transfer/carrier protein, calcium-binding protein, kinase, transferase, and signaling molecule (Table 2). The network of key targets was constructed based on the STRING database (Figure 3(c)). In the network, the key targets interacted with each other through known (from curated databases and experimentally determined), predicted (gene neighborhood, gene fusions, and gene cooccurrence), and other (text mining, coexpression, and protein homology) interactions.

3.6. GO and KEGG Enrichment Analysis. GO functional annotation and KEGG pathway analysis were performed on 119 targets of the PPI network. The top 20 were then visualized as bubble charts (Figure 4). In the biological process, Panax notoginseng has great influence on nutrient levels, lipopolysaccharide, and molecule of bacterial origin (Figure 4(a)). At the molecular level, the function of drug components of Panax notoginseng is mainly related to cytokine receptor binding, receptor ligand activity, and cytokine activity (Figure 4(b)). Targets in the cellular components are closely related to membrane raft, membrane microdomain, and membrane region (Figure 4(c)).

Table 1: Potential effective compounds of Panax notoginseng.

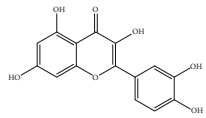
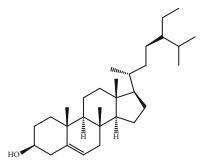
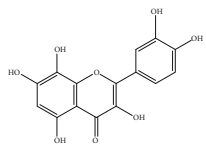
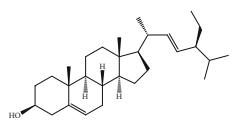
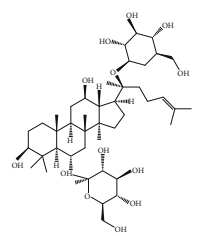
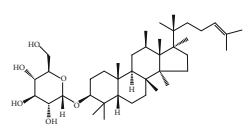
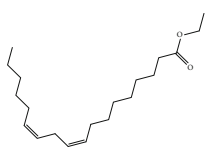
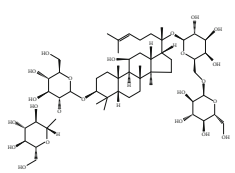
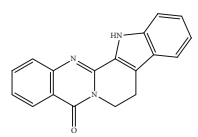
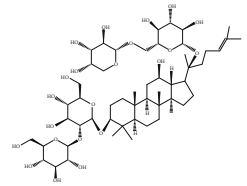
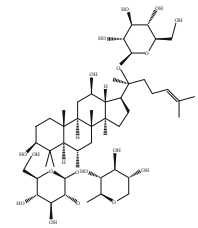
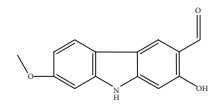
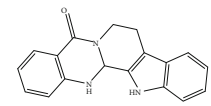
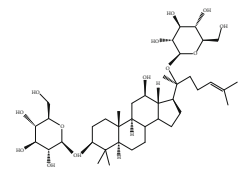
	Compound	OB%	DL	Degree	Structure
1	Quercetin	46.43	0.28	238	
2	Beta-sitosterol	36.91	0.75	121	
3	Gossypetin	35	0.31	102	
4	Stigmasterol	43.83	0.76	96	
5	Ginsenoside Rg1	9.03	0.28	46	
6	Ginsenoside Rh2	36.32	0.56	41	
7	Mandenol	42	0.19	40	
8	Ginsenoside Rb1	6.24	0.04	38	
9	Rutaecarpine	40.3	0.6	36	
10	Ginsenoside Rb2	6.02	0.04	28	

Table 1: Continued.

	Compound	OB%	DL	Degree	Structure
11	Notoginsenoside R1	4.27	0.13	26	
12	2-Hydroxy-3-formyl-7-methoxycarbazole	83.08	0.18	10	
13	Dihydrorutaecarpine	42.27	0.6	4	
14	Ginsenoside F2	36.43	0.25	3	

A total of 230 enrichment results were obtained by KEGG pathway analysis. The first 20 pathways were screened according to adjusted $p < 0.05$ (Figure 5) and consisted of 101 nodes and 419 interaction edges, which mainly involve signaling pathways such as the advanced glycation end products-receptor for advanced glycation end product (AGE-RAGE) signaling pathway in diabetic complications, proteoglycans in cancer, fluid shear stress and atherosclerosis, and interleukin-17 (IL-17) signaling pathway, thus indicating that the effective components of Panax notoginseng might treat MF by acting on these pathways.

3.7. Verification of Results by Molecular Docking. The key targets IL6, ALB, AKT1, TNF, and VEGFA were used for molecular docking with the effective compounds of Panax notoginseng, and a heat map was drawn based on the results (Figure 6). All bioactive components of Panax notoginseng had good binding with key targets, suggesting that Panax notoginseng has a strong tendency as a therapeutic strategy for MF via these key targets.

Results showed that rutaecarpine has a strong binding ability with ALB (docking score = −10.526), AKT1 (docking score = −8.277), and TNF (docking score = −4.689) (Figure 7). Dihydrorutaecarpine has a strong binding ability to IL6 (docking score = −3.345) and ginsenoside Rb1 with VEGFA (docking score = −6.188).

4. Discussion

This study used network pharmacology to systematically analyze the mechanism of action of Panax notoginseng in the treatment of myocardial fibrosis (MF). The resulting PPI network has 119 targets, accounting for one-third of the target number of Panax notoginseng. Five key targets, IL6, ALB, AKT1, TNF, and VEGFA, have high network value. Thus, we speculate that the effective components of Panax notoginseng may have pharmacologic activities in the treatment of MF through these targets.

Fibrosis is the final stage of a chronic inflammatory response, which can be caused by many factors. IL6 is a potent proinflammatory cytokine involved in MF [17]. Fibroblasts maintain this potential pathogenic change by regulating the production of IL6. Overexpression of IL6 is sufficient to induce myofibroblast proliferation, differentiation, and fibrosis. IL6 is involved in ischemic myocardial remodeling by upregulating the TGF-β1 signaling pathway [18–20]. TNF-α is also a proinflammatory cytokine with a wide range of biologic effects and is involved in the pathophysiology of various cardiovascular diseases. Low-level expression of TNF-α in normal myocardium has a protective effect on myocardial cells. However, its increased expression can cause myocardial fibroblast proliferation, myocardial cell death, systolic dysfunction, cardiac fibrosis, and ventricular remodeling [21–23].

In this network pharmacology study, we found that ALB, VEGFA, and AKT1 are also involved in MF. Jäntti et al. found a close relationship between the ALB level and cardiac function. When the plasma ALB level of hemodialysis patients was increased, cardiac function of patients improved, thus effectively raising their quality of life [24]. Studies have found that VEGFA can induce angiogenesis, and in infarcted hearts, VEGFA-mediated cardiac stem cell engraftment resulted in a reduction in fibrosis [25, 26]. The Akt signaling pathway is involved in cardiac hypertrophy

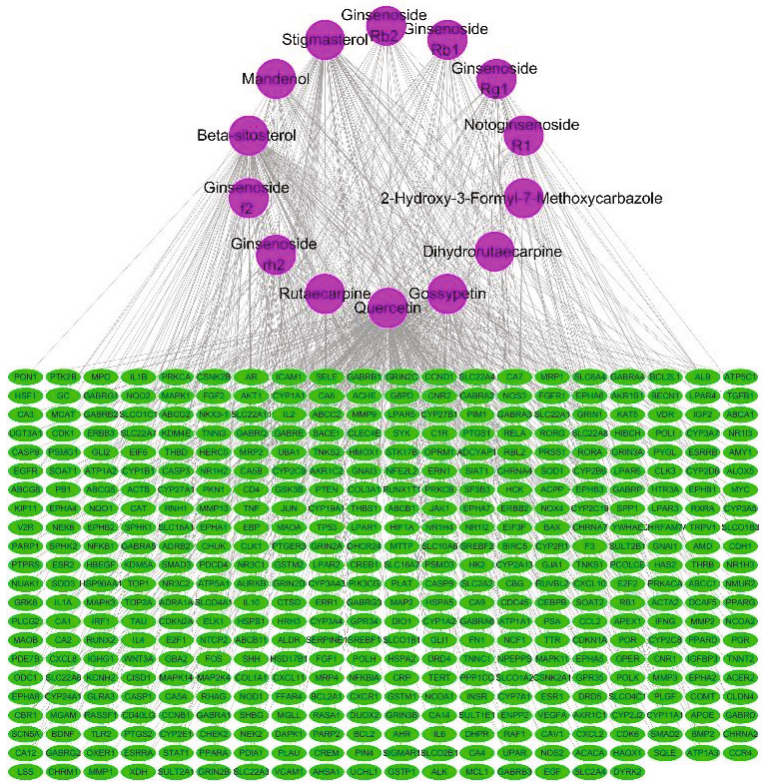


Figure 2: Effective component-target network. Violet nodes represent compounds of Panax notoginseng, and green nodes represent predicted targets.

Table 2: Protein classes of key targets.

Gene name	Target	UniProt ID	Protein class	Degree
IL6	Interleukin 6	P05231	None	106
ALB	Albumin	P02768	Transfer/carrier protein	100
AKT1	AKT serine/threonine kinase 1	P31749	Calcium-binding protein; kinase; transfer/carrier protein; transferase	98
TNF	Tumor necrosis factor	P01375	Signaling molecule	98
VEGFA	Vascular endothelial growth factor A	P15692	Signaling molecule	95

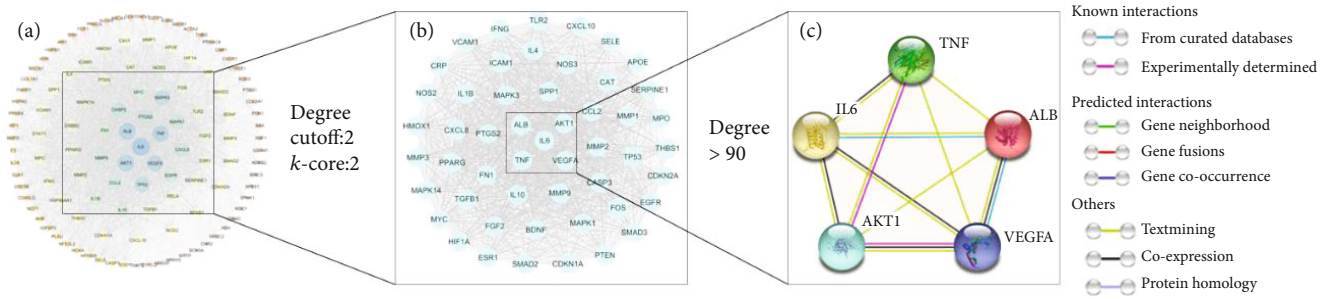
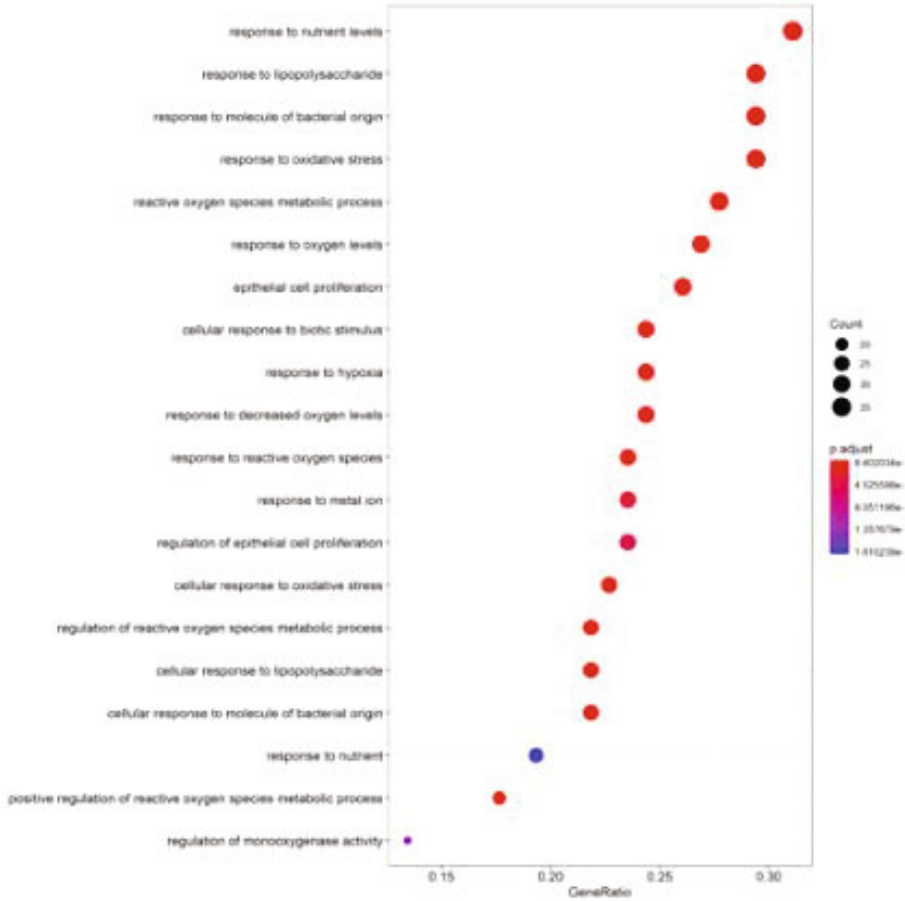
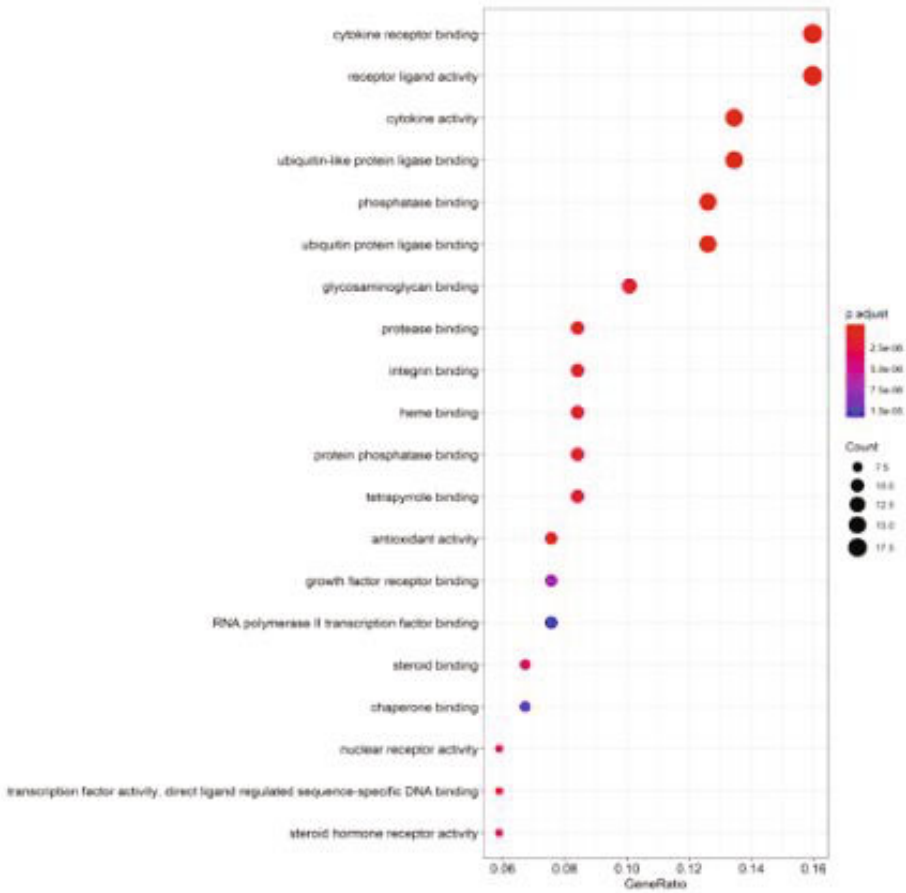


Figure 3: Network diagram of the PPI network, core clusters, and key targets: (a) PPI network; (b) core clusters; (c) key targets.



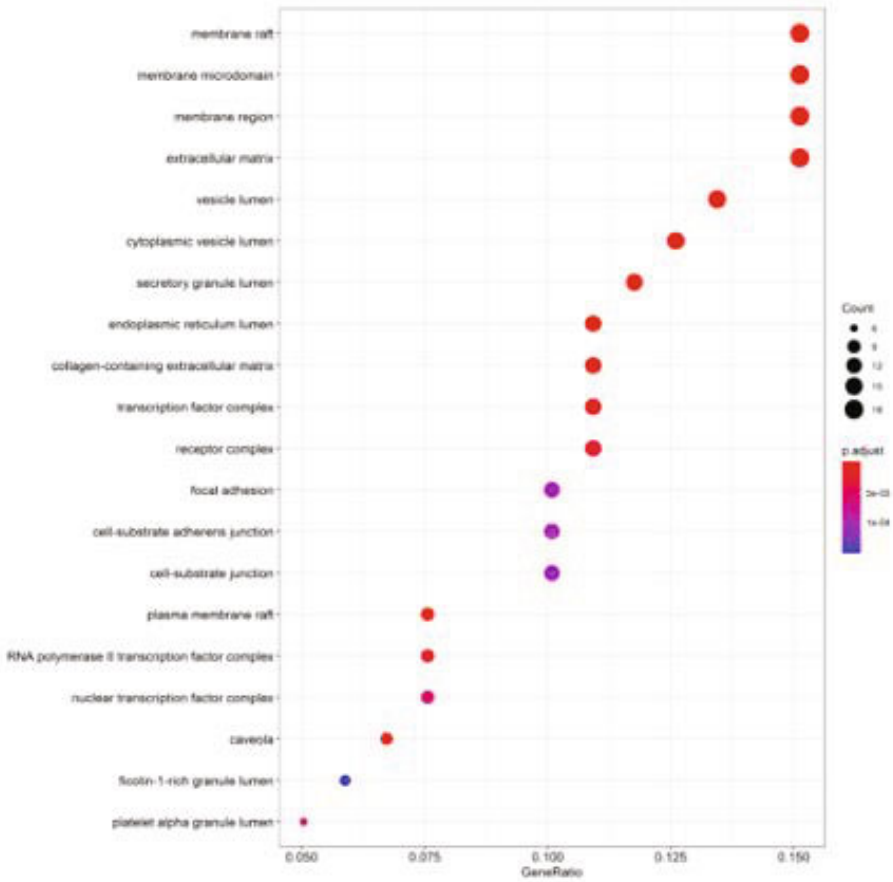
(a)

Figure 4: Continued.



(b)

Figure 4: Continued.



(c)

Figure 4: GO function enrichment analysis of potential targets from active ingredients of Panax notoginseng: (a) biological process; (b) molecular function; (c) cellular component.

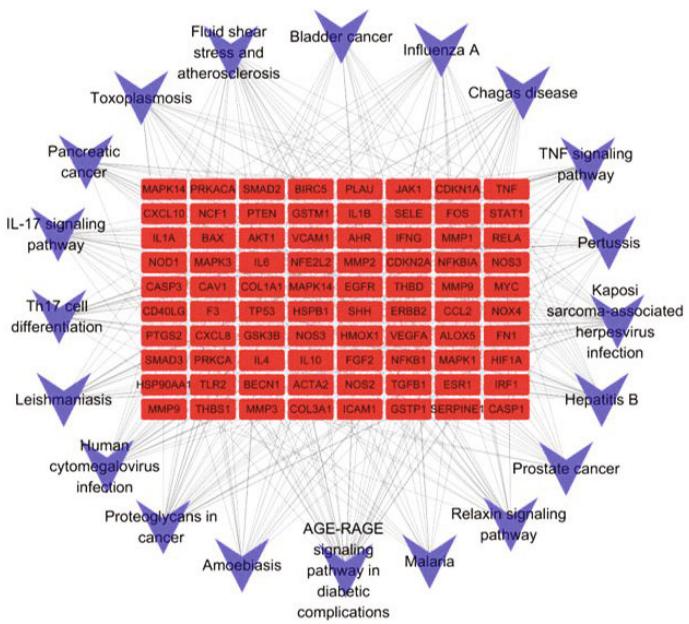


Figure 5: Target-KEGG pathway network. Blue nodes represent 20 KEGG pathways, and red nodes represent common targets.

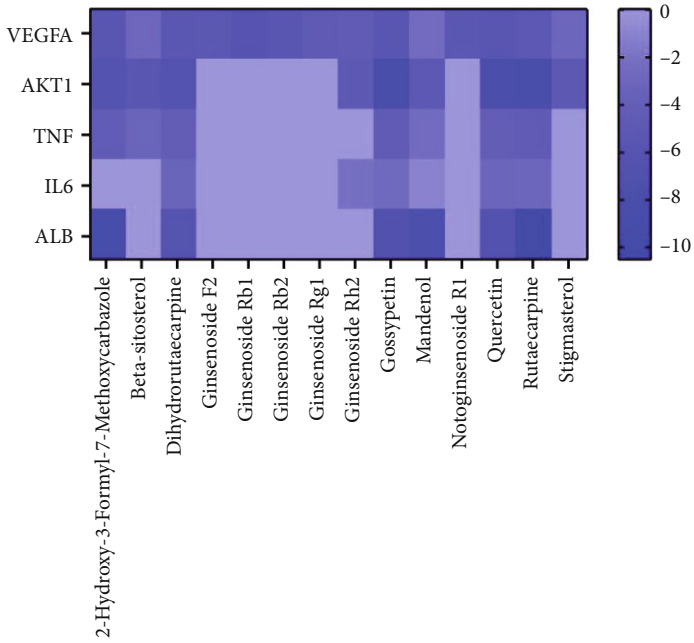


Figure 6: Heat maps of the docking scores of key targets combining with bioactive compounds in Panax notoginseng.

and remodeling. Short- and medium-term overexpression of AKT1 leads to physiologic hypertrophy, but long-term activation of AKT1 can lead to pathologic hypertrophy, such as systolic dysfunction [27]. Knockdown of AKT1 in macrophages can reduce transdifferentiation of fibroblasts, suggesting that AKT1, as an important signaling molecule, may regulate fibroblast transdifferentiation by promoting an inflammatory reaction [28].

Through this network pharmacology study, we found that Panax notoginseng in treating MF mainly involves the AGE-RAGE signaling pathway in diabetic complications, proteoglycans in cancer, and IL-17 signaling pathways. Cardiovascular complications are the leading cause of death in diabetic patients. The AGE-RAGE signaling pathway of myocardial fibrosis in diabetic complications has been widely studied. It regulates the pathogenesis of cardiovascular disease and promotes increased collagen deposition leading to tissue fibrosis [29, 30]. Therefore, targeting the AGE-RAGE signaling pathway is a potential therapeutic strategy for ameliorating CVDs in diabetes.

Recent studies have shown that proteoglycans are promising diagnostic biomarkers for cardiac fibrosis and may provide new treatment strategies for heart disease [31]. Proteoglycans are a nonstructural component of the extracellular matrix and regulate many aspects of the immune response [32, 33]. Decorin, a well-investigated proteoglycan, inhibits both bioactivity and gene expression of TGF-β, a powerful fibrogenic factor [34]. Furthermore, decorin gene transfer significantly attenuates interstitial fibrosis and cardiac hypertrophy [35]. In various forms of cardiac fibrosis, the expression of the four-membered family of transmembrane proteoglycans, syndecan-1 to syndecan-4, is upregulated in response to proinflammatory stimuli and regulating fibrosis [36].

IL-17 has also been found to be involved in MF. In diabetic mice, IL-17 can reduce MF and improve cardiac function by inhibiting long-term noncoding RNA-AK081284 [37]. Studies have found that IL-17 contributes to MF through the protein kinase C (PKC) β/Erk1/2/NF-κB (nuclear factor κappa B) pathway [38, 39]. Thus, it can be inferred that AGE-RAGE, proteoglycans, IL-17, and other signaling pathways appear to be closely related to the mechanism of Panax notoginseng in the treatment of MF.

We found that rutaecarpine and ginsenoside Rb1 are the prime binding compounds to the key targets through molecular docking. Rutaecarpine exhibits a number of pharmacologic effects on the cardiovascular system including cardiac protective, vasodilator, and antiatherosclerotic activities [40, 41]. Rutaecarpine has been found to significantly improve cardiac function and decrease the content of TNF-α in myocardial tissues [42]. Ginsenoside Rb1, an active saponin of Panax notoginseng, has anti-inflammatory and antioxidative functions. It decreases the heart rate, improves cardiac function, and attenuates histologic changes induced by heart failure. Furthermore, ginsenoside Rb1 has also been shown to protect cardiomyocytes by targeting microRNA-21 and reverse the imbalance between apoptosis and autophagy in atherosclerosis [43–45]. While research on the above compounds has revealed their potential effects on the heart muscle, the specific mechanisms of their antimyocardial fibrotic actions remain unclear and need further study.

5. Conclusion

Panax notoginseng has a wide range of clinical applications in treating MF, but there are few reports on its pharmacologic activities. In this network pharmacology study, a multicomponent, multitarget, and multipathway treatment of MF

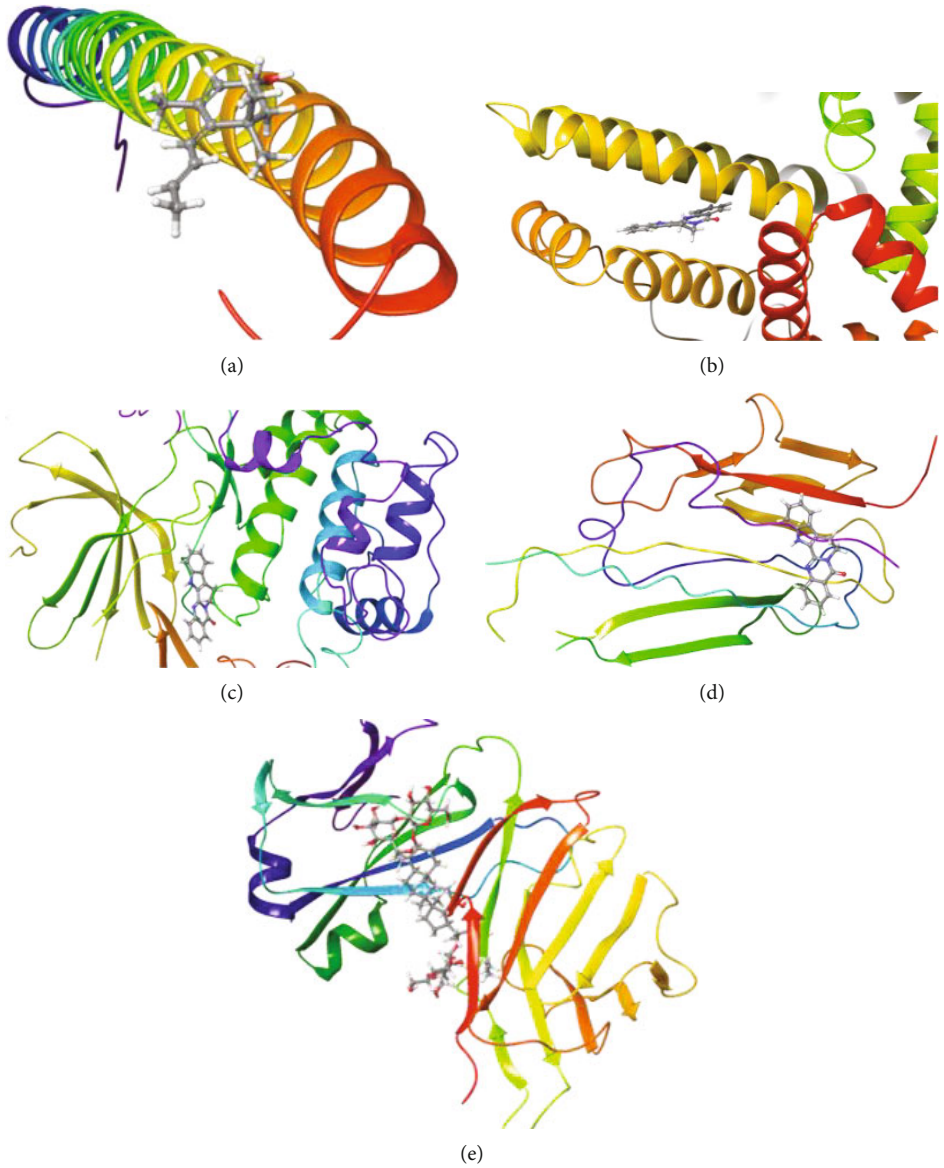


Figure 7: Molecular docking simulation of bioactive compound-key target: (a) IL6-dihydrorutaecarpine; (b) ALB-rutaecarpine; (c) AKT1-rutaecarpine; (d) TNF-rutaecarpine; (e) VEGFA-ginsenoside Rb1.

using Panax notoginseng was established and provides a theoretical basis for clinical treatment of MF. However, the results of this study are based on data analysis and have only a certain predictive effect, which needs to be verified by further in vitro and in vivo experiments.

Data Availability

The data used to support the findings of the study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Tingting Zhao and Xinwei Wang conceived and designed the study. Jingxue Han, Jingyi Hou, Yu Liu, and Peng Liu performed the data analysis. Jingxue Han wrote the paper. All authors have read and approved the final manuscript.

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Research Article

Association of HLA-B Gene Polymorphisms with Type 2 Diabetes in Pashtun Ethnic Population of Khyber Pakhtunkhwa, Pakistan

Asif Jan,[1] **Muhammad Saeed**,[1] **Muhammad Hussain Afridi**,[2] **Fazli Khuda**,[1] **Muhammad Shabbir**,[3] **Hamayun Khan**,[1] **Sajid Ali**,[4] **Muhammad Hassan**,[5] **Samiullah**,[1] **Rani Akbar**,[6] **and Zakiullah**[1]

[1]*Department of Pharmacy, University of Peshawar, Pakistan*
[2]*Diabetes and Endocrinology Unit, Hayatabad Medical Complex Peshawar, Pakistan*
[3]*Internal Medicine, College of Medicine, Shaqra University, Saudi Arabia*
[4]*Department of Biotechnology, Abdul Wali Khan University, Mardan, Pakistan*
[5]*Pakistan Air Force Hospital, Fort Munro, Pakistan*
[6]*Department of Pharmacy, Abdul Wali Khan University, Mardan, Pakistan*

Correspondence should be addressed to Zakiullah; zakiullah@uop.edu.pk

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Human leukocyte antigen (HLA) system is the most polymorphic and gene dense region of human DNA that has shown many disease associations. It has been further divided into HLA classes I, II, and III. Polymorphism in HLA class II genes has been reported to play an important role in the pathogenesis of type 1 diabetes (T1D). It also showed association with T2D in different ethnic populations. However, a little is known about the relationship of HLA class I gene polymorphism and T2D. This study has evaluated the association of HLA-B (class I gene) variants with T2D in Pashtun ethnic population of Khyber Pakhtunkhwa. In the first phase of the study, whole exome sequencing (WES) of 2 pooled DNA samples was carried out, and DNA pools used were constructed from 100 diabetic cases and 100 control subjects. WES results identified a total of $n = 17$ SNPs in HLA-B gene. In the next phase, first 5 out of $n = 17$ reported SNPs were genotyped using MassARRAY® system in order to validate WES results and to confirm association of selected SNPs with T2D. Minor allele frequencies (MAFs) and selected SNPs×T2D association were determined using chi-square test and logistic regression analysis. The frequency of minor C allele was significantly higher in the T2D group as compared to control group (45.0% vs. 13.0%) ($p = 0.006$) for rs2308655 in HLA-B gene. No significant difference in MAF distribution between cases and controls was observed for rs1051488, rs1131500, rs1050341, and rs1131285 ($p > 0.05$). Binary logistic regression analyses showed significant results for SNP rs2308655 (OR = 2.233, CI (95%) = 1.223-4.077, and $p = 0.009$), while no considerable association was observed for the other 4 SNPs. However, when adjusted for these variants, the association of rs2308655 further strengthened significantly (adjusted OR = 7.485, CI (95%) = 2.353-23.812, and $p = 0.001$), except for rs1131500, which has no additive effect. In conclusion, the finding of this study suggests rs2308655 variant in HLA-B gene as risk variant for T2D susceptibility in Pashtun population.

1. Introduction

Type 2 diabetes (T2D) is a multifactorial metabolic disease characterised by impaired glucose haemostasis that is primarily caused by lack of response of peripheral tissues to insulin and/or insufficient production/secretion of insulin by β cells of pancreas. Environmental and genetic variations are key risk factors for T2D [1, 2]. According to the recent report (Diabetes Atlas edition 2019) of International Diabetes Federation (IDF), approximately 415 million people around the world have diabetes, with 90% of these individuals having T2D [3]. Currently, the epicentres of diabetes

mellitus (DM) prevalence have been in China, India, United States of America (USA), Pakistan, and Brazil [4]. Its prevalence rate is alarmingly high in developing countries, with more than 80% cases being reported in these nations [5]. It is projected that by 2045, the number of cases in Pakistan will exceed that of in the USA, moving the former from 4th position to 3rd in diabetes prevalence race [3]. Currently in Pakistan, 19.4 million people are living with diabetes [3]; the number was 5.5 million in 2000 [6].

Type 2 diabetes (T2D) risk is strongly heritable [7–9]. Genetic studies offer a powerful approach for better screening and treatment of diseases by identifying alterations at molecular level associated with physiological trait [10, 11]. Till date, a rich landscape of information about pathogenesis of T2D has been provided [12, 13]. Recent genome-wide association studies (GWAS) in different ethnic population around the world have identified hundreds of T2D susceptible genomic variants, although translating these findings into clinical practice is still challenging [7, 14]. Noteworthy, *CDKAL1*, *HLA-B*, *TCF7L2*, *SLC30A8*, *HHEX*, *IGF2BP2*, *CDKN2A/B*, *EXT2*, and *FTO* genes were found to be associated with T2D in different ethnic populations around the world [15–21]. Very few such studies on T2D are available in Pakistani cohort. Genes like *HHEX/IDE*, *KCNJ11*, *NOTCH2*, *WFS1*, *IRS1*, *CAPN10*, *KCNQ1*, *HNF4A*, *TCF-2/HNF1B*, *IRS-2*, and *TCF7L2* have been studied to be associated with T2D in Pakistani population [22–26].

Pakistani population includes 5 major ethnic groups, namely, Punjabis, Pashtuns, Sindhis, Baluchis, and Muhajirs. Pashtun constitute the major population of Khyber Pakhtunkhwa (KP). They have unique cultural practices and social values, life style, and behaviours that make them a suitable population for such studies. It is hypothesized that genetic mutation spectrum of type 2 diabetes in Pakistani population is different from elsewhere [23, 27]. This study has found deleterious mutations in human leukocyte antigen-B (HLA-B) gene associated with type 2 diabetes in Pashtun ethnic population of Khyber Pakhtunkhwa, Pakistan, using high-throughput sequencing.

The study will help to better understand the pathogenesis of T2D in the study population and to devise modification strategies to overcome/control the burden of this fatal and costly disease.

2. Materials and Methods

2.1. Participants. A total of 200 individuals (diabetic $n = 100$ and nondiabetic $n = 100$) of Pashtun ethnicity belonging from different districts such as Peshawar, Charsadda, Mardan, Bannu, Kohat, Dir, and Swat of Khyber Pakhtunkhwa were included in the study. Patients were registered at Lady Reading Hospital (LRH) Peshawar, Hayatabad Medical Complex (HMC) Peshawar, and Khyber Teaching Hospital (KTH) Peshawar while control samples were collected from specially organized diabetes free medical and screening camps at Rehman Medical Institute (RMI) Hayatabad Peshawar and Diabetic Association Charsadda (DAC). The study period was from July 2018 to July 2019. Inclusion criteria for cases were (i) diabetes diagnosed as per International Diabetes Federation (IDF) etiologic classification, (ii) confirmation that subjects are of Pakistani origin and Pashtun ethnic, and (iii) age above 30 years. Exclusion criteria were (i) mentally ill patients, (ii) age below 30 years, and (iii) diabetes during pregnancy, recent infections, and presence of malignancies. Patient's inclusion and exclusion were according to the previously defined criteria used for Asian populations [28]. Control subjects were healthy individuals from general population with blood sugar level in normal range (<99 mg/dL fasting or 120 mg/dL) [29]. Consent form and thorough demographic, family, and clinical history of all the participants were taken on a carefully designed Proforma. For illiterate participants, who did not understand English, consent form for their understanding was read and explained in local Pashtu language and then signed on his behalf by any of his/her relative/attendant. The study protocols were according to the guidelines of Helsinki declaration (1975), and ethical approval was obtained from the Ethical Committee of the Department of Pharmacy, University of Peshawar. For overall study design, see Figure 1.

2.2. Blood Sampling. Three-millilitre whole blood was collected following aseptic procedures from the median cubital vein of study individuals in EDTA tubes (properly labelled) and was stored at -10°C.

2.3. DNA Extraction and Pool Preparation. DNA was extracted from 200 μl whole blood samples of T2D patients using WizPrep DNA extraction kit (WizPrep no. W54100). DNA quantification was carried with the help of Qubit™ dsDNA HS Assay kit (Catalog No. Q32851) using Introgen Qubit™ 3, and concentration was adjusted to 10 ng/μL.

2.4. Whole Exome Sequencing. Whole exome sequencing (WES) was carried out at the Centre of Genomics, Rehman Medical Institute (RMI), Hayatabad, Peshawar. In order to simplify sequencing process and to reduce cost and time, DNA pools were constructed from 100 diabetic cases and 100 control subjects according to the previously described DNA-pooling protocols [30, 31]. Each pool contains an equimolar amount of DNA (100 ng) from each individual. DNA pools were then subjected to amplification and sequencing via HiSeq2500 platform (Illumina, San Diego, CA, USA) using paired-end libraries (2×101 bp).

2.5. WES Analysis. A custom-built in-house NGS bioinformatics pipeline was employed in order to move raw sequencing data to final variant calls. Raw FASTQ files produced by the Illumina HiSeq were filtered to separate low quality reads ($Q > 30$) using CASAVA and trimmomatic tool [32, 33]. The filtered reads were then aligned to the reference genome (hg19/GRCh37) using BWA-mem (v 0.7.13) [34, 35]. Base recalibration was carried using GATK (v 3.2.2). Variants were called using GATK Unified Genotyper; the called variants were stored as VCF file. The variants were annotated from variant calling file using ANNOVER [36]. The resulting annotated variant file was loaded in Excel program file for easy understanding, filtering, and analysis of data.

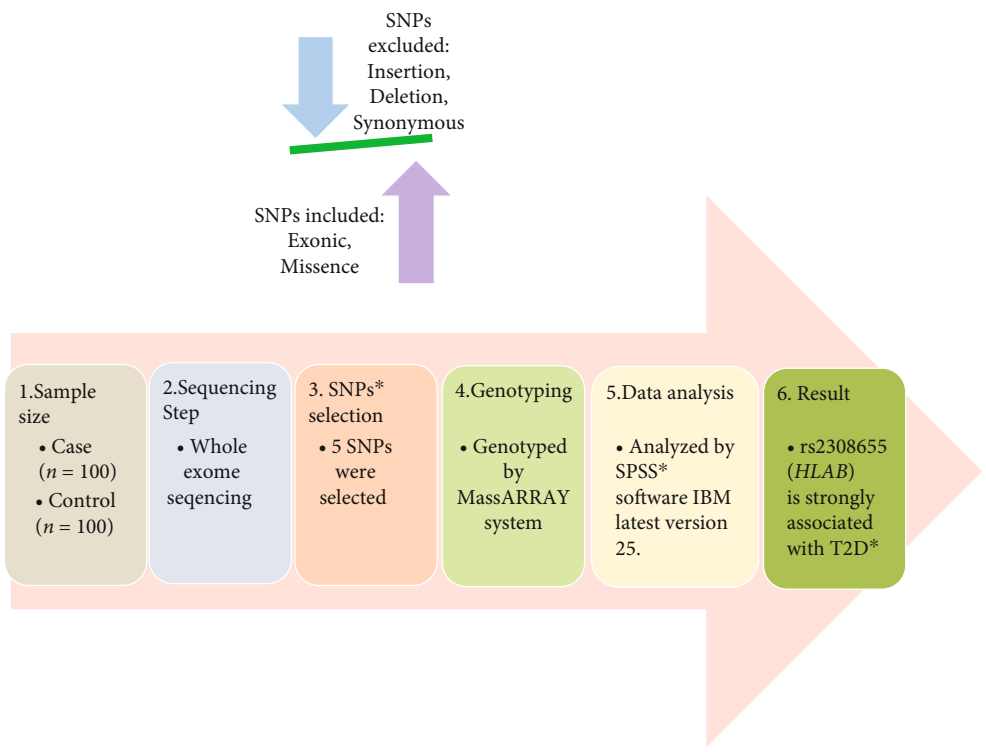

Figure 1: Flow chart of overall study design. SNPs: single-nucleotide polymorphisms; T2D: type 2 diabetes; HLA-B: human leukocyte antigen-B gene.

2.6. WES Results. Exome sequencing identified a total of 1048575 SNPs including 607572 homozygous SNPs, 441003 heterozygous SNPs, 99392 deletion, 74390 insertion, 50280 exonic SNPs, 7710 missense variants, 1797 variants expressed in pancreas, and 570 possibly pathogenic mutations. Detailed WES results are shown in Figure 2.

2.7. Filtration of WES Data. In search of potential T2D, associated variant data was filtered. The flow diagram of data filtration is shown in Figure 3. The annotated Excel files were first manually curated to shortlist exonic and missense variants while synonymous variants were discarded. Resultant file was filtered for T2D susceptible genes (supplementary file 1). HLA-B gene was found to be of interest to be further investigated, among several others. Reported HLA-B variants ($n = 17$) are shown in Table 1.

2.8. Validation Trail and Genotyping of HL-B Variants. A total of $n = 17$ SNPs in HLA-B gene were identified using whole exome sequencing (Table 1). In order to validate whole exome sequencing results and to affirm the association of the newly identified HLA-B risk variants with T2D, SNPs were genotyped. Out of $n = 17$ reported SNPs, first 5 SNPs (rs2308655, rs1051488, rs1131500, rs1050341, and rs1131285) were selected for further study. Genotyping of the selected candidate SNPs was carried at the Centre of Genomics, Rehman Medical Institute (RMI), Hayatabad, Peshawar, using Sequenom MassARRAY® system (Agena Bioscience, San Diego, CA) carefully following the manufacturer's guidelines.

2.9. Statistical Analysis. Statistical data analysis was performed using IBM SPSS (Statistical Package for Social Sciences version 24). Key variables selected for analysis were age, gender, weight, geographical area, life style, smoking, exercise, occupation, diet, and variants in HLA-B gene. Quantitative variables were checked using independent samples t-test. Data for quantitative variables were shown as mean ± standard deviation, while categorical variables of cases and controls were compared using chi-square (χ^2) test. Data for categorical variables were expressed as number and percentage. All reported SNPs were tested for Hardy-Weinberg equilibrium (HWE). Minor allele frequencies (MAFs) between cases and controls were compared using χ^2 test. The association of selected individual SNPs×T2D was checked using binary logistic regression and was also adjusted for combinations within themselves to determine their combined effect. Statistically, a $p \leq 0.05$ was considered significant.

3. Results

3.1. Subject Characteristics. Demographic and general characteristics of study subjects are shown in Tables 2 and 3. The prevalence of comorbidities like hypertension, ischemic heart disease, renal failure, retinopathy, and hypercholesterolemia was higher in cases than those of control subjects as shown in Table 2. No significant difference (t-test $p = 0.104$) in mean weight of cases and controls (62.64 ± 6.07 vs. 59.55 ± 8.32) was observed. An average normal blood pressure (120/80 mmHg) was observed in patients with T2D; however, an elevated blood pressure (>120/80 mmHg) was observed in patients with comorbidities like ischemic heart

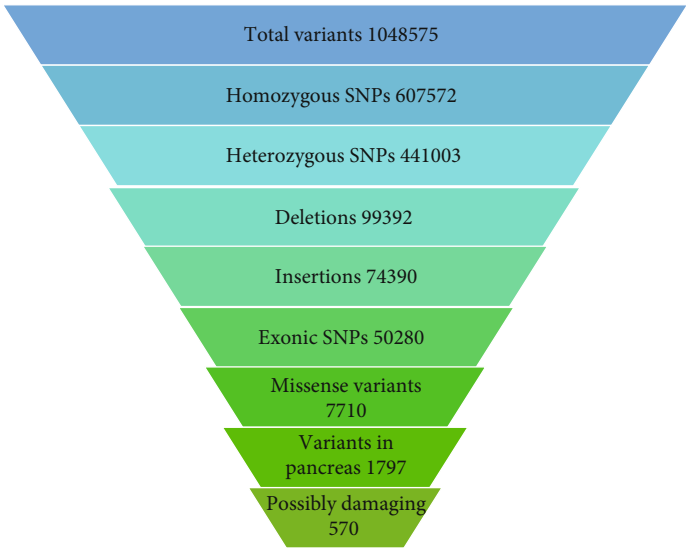

Figure 2: Whole exome sequencing results of study subjects.

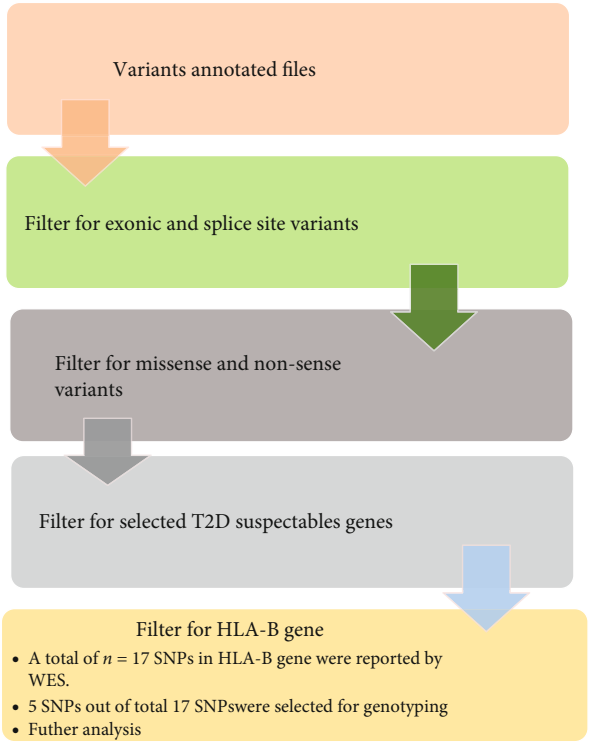

Figure 3: Variant filtration and prioritization pipeline. SNPs: single-nucleotide polymorphisms; T2D: type 2 diabetes; HLA-B: human leukocyte antigen-B gene.

disease, renal failure and hypercholestermia. Reference to the patient's general characteristics is shown in Table 3. Sixty-five percent (65%) of patients were males, and 35% were females. The highest incidence of T2D was reported in district Peshawar (53%) followed by district Charsadda (13%), district Mardan (12%), district Swat (08%), district Kohat (06%), district Dir (05%), and district Bannu (03%). Patients included in the study were from different occupations, and most of the patients were doing laborious jobs like driving and farming. Occupation wise, 34% female patients were housewives, 20% were labours, 16% were businessmen, 13% were government servants, 10% were drivers, while 07% were farmers. The highest (34%) prevalence was seen in female patients who were housewives. When patients were asked for family history of T2D, 94% answered "Yes" for family of diabetes while 06% answered "No" for family history of diabetes. Moreover, 85% of patients were nonexercising (sedentary life style), 15% were exercise loving, and none of the patient (0%) was attached with any sport or gym. Majority of (60%) patients were nonsmokers, 21% of patients were using Naswar (a local smokeless tobacco product), while 19% were chain cigarette smokers. The fraction of patients who were taking proper diet in order control diabetes and its complication was 50% while the rest (50%) showed no diet compliance habits.

3.2. Minor Allele Frequency (MAF) Analysis. The minor allele frequencies for rs2308655, rs1051488, rs1131500, rs1050341, and rs1131285 were compared between T2D group ($n = 100$) and control group ($n = 100$) using chi-square test. The frequency of the minor allele C was significantly higher in the T2D group as compared to control group (45.0% vs. 13.0%; $p = 0.006$) for rs2308655 in HLA-B gene, while no significant differences in minor allele distribution between cases and control were observed for the other 4 SNPs. For complete detail of MAF comparison between cases and controls, consider Table 4.

3.3. Association between SNPs and T2D (SNPs×T2D). All 5 HLA-B variants (rs2308655, rs1051488, rs1131500, rs1050341, and rs1131285) were checked for T2D association using logistic regression analysis. The frequency of minor C allele was significantly higher in the T2D group as compared to control group (45.0% vs. 13.0%) ($p = 0.006$) for rs2308655 in HLA-B gene. No significant difference in MAF

Table 1: HLA-B variants ($n = 17$) reported by whole exome sequencing.

SNP ID	Gene	Variant	Chr	cDNA position	Protein position	HGVSc	HGVSp	SIFT score	PolyPhen score	Alternate allele frequency (%)		Read depth	
										Case	Control	Case	Control
rs2308655	HLA-B	C>C/G	6	1100	349	c.1046G>C	p.Cys349Ser	Del (0.03)	Damaging	87.48	55.22	235	169
rs1051488	HLA-B	C>C/T	6	1039	329	c.985G>A	p.Ala329Thr	Tol (0.05)	Benign	49.07	45	108	80
rs1131500	HLA-B	C>C/T	6	970	306	c.916G>A	p.Val306Ile	Tol (1)	Benign	47.35	49.07	77	54
rs1050341	HLA-B	T>T/C	6	706	218	c.652A>G	p.Ile218Val	Tol (0.51)	Benign	52.16	53.02	301	218
rs1131285	HLA-B	C>C/G	6	664	204	c.610G>C	p.Glu204Gln	Tol (1)	Benign	83.33	85.19	228	257
rs1131275	HLA-B	G>G/C	6	657	201	c.603C>G	p.Asp201Glu	Tol (1)	Benign	85.24	86.05	244	258
rs1050696	HLA-B	A>A/G	6	637	195	c.583T>C	p.Tyr195His	Tol (0.22)	Benign	24.73	29.9	275	291
rs2308466	HLA-B	T>G/A	6	614	187	c.560A>C	p.Glu187Ala	Tol (0.57)	Benign	34.67	41.52	349	289
rs2523600	HLA-B	C>T/G	6	613	187	c.559G>A	p.Glu187Lys	Tol (0.71)	Benign	34.67	41.32	349	288
rs697742	HLA-B	C>C/A	6	593	180	c.539G>T	p.Arg180Leu	Tol (0.66)	Benign	50.99	59.2	402	299
rs9266144	HLA-B	G>G/A	6	592	180	c.538C>T	p.Arg180Trp	Tol (0.2)	Benign	16.34	19.4	410	299
rs151341293	HLA-B	T>T/A	6	581	176	c.527A>T	p.Glu176Val	Tol (0.65)	Benign	25.11	33.04	462	339
rs1050654	HLA-B	G>G/T	6	517	155	c.463C>A	p.Arg155Ser	Tol (0.09)	Benign	37.63	53.15	481	301
rs1140412	HLA-B	G>G/C	6	417	121	c.363C>G	p.Ser121Arg	Tol (0.79)	Benign	61.79	68.91	123	119
rs1071652	HLA-B	C>C/G	6	416	121	c.362G>C	p.Ser121Thr	Tol (0.75)	Benign	61.67	59.02	120	122
rs1050388	HLA-B	C>C/T	6	356	101	c.302G>A	p.Ser101Asn	Tol (1)	Benign	22.08	29.59	240	169
rs1131215	HLA-B	C>C/A	6	346	98	c.292G>T	p.Asp98Tyr	Tol (0.62)	Benign	74.04	72.97	262	185

Abbreviations: SNP: single nucleotide polymorphism; Chr: chromosome; HGVS: human genome association variation; HGVSc: the HGVS coding sequence name; HGVSp: the HGVS protein sequence name; Del: deletion; Tol: tolerated.

Table 2: Comorbidities prevalence in study subjects.

Disease	Frequency	
	Cases	Control
Hypertension	34.00%	10.00%
IHD	14.00%	0.00%
Renal failure	5.00%	0.00%
Retinopathy	61.00%	0.00%
Hypercholesterolemia	6.00%	3.01%
HCV	1.00%	0.00%
HBV	0.00%	0.00%

IHD: ischemic heart disease; HCV: hepatitis C virus; HBV: hepatitis B virus.

distribution between cases and controls was observed for rs1051488, rs1131500, rs1050341, and rs1131285 ($p > 0.05$). Binary logistic regression analyses showed significant association for SNP rs2308655 (OR = 2.233, CI (95%) = 1.223-4.077, and $p = 0.009$), while no considerable association was observed for the other 4 SNPs. However, when adjusted for these variants, the association of rs2308655 further strengthened significantly (adjusted OR = 7.485, CI (95%) = 2.353-23.812, and $p = 0.001$), except for rs1131500, which has no additive effect. For detailed statistical analyses, see Table 5.

4. Discussion

The human leukocyte antigen (HLA) gene complex encodes the major histocompatibility complex (MHC) in humans and linked with numerous diseases [37–40] (supplementary file 2). It has three main classes: class I HLAs (A, B, and C), class II HLAs (DQ, DM, DP, DR, and DO), and class III HLAs (CSK2B, SKI2W, C4B, and PBX2) [41]. Mutations in HLA class II alleles are linked with T2D according to previous studies [42–44]. A meta-analysis of 17 genome-wide association studies on T2D performed in African American cohort linked polymorphism in HLA-B (class I gene) with T2D [45]. HLA-B not only increase disease susceptibly but also involve in many adverse drug reaction [46–49]. The present study evaluates the association of genetic variations in HLA-B, a highly polymorphic gene with >3000 variants [50] with T2D in Pashtun ethnic population of Khyber Pakhtunkhwa, Pakistan, using whole exome sequencing.

We performed whole exome sequencing of pooled DNA samples and genotyping of selected SNPs using MassARRAY® system to identify T2D risk variants. Using this approach, our first top notable candidate SNP reported was missense, heterozygous variant rs2308655 (c.1046G>C, p.Cys349Ser) located on short arm of chromosome 6. SIFT and PolyPhen (scores 0.03 and 0.991) predicated rs2308655 variant as deleterious and probably damaging. Second missense, heterozygous, exonic variant reported was rs1051488 (c.985G>A, p.Ala329Thr). SIFT and PolyPhen (scores 0.05 and 0.062) predicated rs2308655 variant as tolerated low confidence and of benign nature. A third missense mutation rs1131500 (c.916G>A, p.Val306Ile) reported was tested nondamaging and benign by SIFT and PolyPhen (scores of 1.00 and 0.001). The last two common missense variants reported were rs1050341 (c.652A>G, p.Ile218Val) and rs1131285 (c.610G>C, p.Glu204Gln); both variants were scored nondamaging and benign by SIFT and PolyPhen.

Table 3: Sociodemographic features of cases and controls.

Variables	Case $n(f)$	Control $n(f)$	p value
Gender			
Male	65 (65.0%)	77 (77.0%)	0.061
Female	35 (35.0%)	23 (23.0%)	
Mean age (yrs)	58 ± 12.40	56 ± 13.43	0.951
Mean weight (kg)	62.64 ± 6.07	59.55 ± 8.32	0.104
Occupation			
Labour	20 (20.0%)	14 (14.0%)	
Govt servant	13 (13.0%)	21 (21.0%)	
Business man	16 (16.0%)	18 (18.0%)	0.112
Farmer	07 (7.00%)	16 (16.0%)	
Housewife	34 (34.0%)	23 (23.0%)	
Driver	10 (10.0%)	08 (8.00%)	
Geographical area (district)			
Peshawar	53 (53.0%)	19 (19.0%)	
Charsadda	13 (13.0%)	53 (53.0%)	
Swat	08 (8.00%)	07 (7.00%)	0.145
Dir	05 (4.00%)	05 (5.00%)	
Mardan	12 (12.0%)	10 (10.0%)	
Kohat	06 (6.00%)	03 (3.00%)	
Bannu	03 (3.00%)	03 (3.00%)	
Family history of T2D			
Yes	94 (94.0%)	0.0 (0.00%)	0.01
No	06 (6.00%)	100 (100%)	
Exercise			
Nonexercising	85 (85.0%)	89 (89.0%)	
Walking	14 (14.0%)	04 (4.00%)	0.016
Jogging	01 (1.00%)	05 (5.00%)	
Gym/sport	0.0 (0.0%)	02 (2.00%)	
Smoking			
Cigarette	19 (19.0%)	10 (10.0%)	0.178
Snuff	21 (21.0%)	26 (26.0%)	
Nonsmoking	60 (60.0%)	64 (64.0%)	
Diet control/compliance			
Yes	50 (50.0%)	90 (90.0%)	0.03
No	50 (50.0%)	10 (10.0%)	

kg: kilogram; yrs: years; T2D: type 2 diabetes; $n(f)$: number (frequency).

Our study pinpoints factors like genetic mutations, ethnicity, and environmental and demographic differences involved in the incidence of T2D. The MHC is regarded as highly gene dense and hyperpolymorphic region of human genome [41, 51]. Gene's mutation in MHC region is involved in pathogenesis of diseases like diabetes [52], rheumatoid arthritis [53], cancer [54], multiple sclerosis [55], and psoriasis [56] and also involved in adverse drug reactions [57, 58]. We tested association of genetic polymorphism in HLA-B and T2D susceptibly in Pashtun ethnic population of Khyber Pakhtunkhwa, Pakistan. A large meta-analysis in African American cohort confirms association of T2D and

Table 4: Minor allele frequency comparison between cases and controls.

SNP	Chr(gene)	Minor allele	Minor allele frequency (%)		p value*
			T2D ($n = 100$)	Control ($n = 100$)	
rs2308655	6(HLA-B)	C	45.0%	13.0%	0.006
rs1051488	6(HLA-B)	T	49.0%	45.0%	0.396
rs1131500	6(HLA-B)	T	47.0%	49.0%	0.572
rs1050341	6(HLA-B)	T	48.0%	47.0%	0.888
rs1131285	6(HLA-B)	C	17.0%	15.0%	0.7

*Chi-square test; $p < 0.05$ is considered significant.

Table 5: Association between selected 5 single-nucleotide polymorphisms and T2D.

SNP	Chr(gene)	Minor allele	OR	CI (95%)	p value*
rs2308655	6(HLA-B)	C	2.233	1.233-4.077	0.009
rs1051488	6(HLA-B)	T	1.301	0.745-2.272	0.355
rs1131500	6(HLA-B)	T	1.199	0.687-2.092	0.522
rs1050341	6(HLA-B)	T	0.98	0.562-1.709	0.944
rs1131285	6(HLA-B)	C	1.17	0.537-2.549	0.692
Adjusted odd ratio					
rs2308655	6(HLA-B)	C	7.485	2.353-23.812	0.001
rs1051488	6(HLA-B)	T	0.574	0.00-0.010	0.999
rs1131500	6(HLA-B)	T	1.779	0.00-0.003	1
rs1050341	6(HLA-B)	T	0	0.01-0.002	0.999
rs1131285	6(HLA-B)	C	0.484	0.177-1.322	0.157

*In binary regression analysis: $p < 0.05$ is considered significant; OR: odd ratio; CI (95%): 95% confidence interval; T2D: type 2 diabetes.

HLA-B polymorphism [45]. Our results suggest strong association of rs2308655 variant in HLA-B gene with T2D. MAF comparison analysis for rs2308655 variant shows that the frequency of minor allele C is much higher in cases than in controls ($p = 0.006$). Regression analysis of rs2308655 also showed significant results (OR = 2.233, CI (95%) = 1.223-4.077, and $p = 0.009$). In contrast, the other 4 SNPs (rs1051488, rs1131500, rs1050341, and rs1131285) were salient showing no association with T2D. Binary regression analysis results for rs1051488, rs1131500, rs1050341, and rs113128 in HLA-B region were found nonsignificant. However, these genes potentiate the effect of rs2308655, as odd ratios increased significantly when these variants were adjusted with it (adjusted OR = 7.485, CI (95%) = 2.353-23.812, and $p = 0.001$) (for details, consider Tables 3 and 4).

The exact underlying mechanism of HLA-B polymorphism and incidence of T2D is not yet known, and comprehensive studies on large scale are needed to explore the mechanism rs2308655 variant in T2D.

Furthermore, sociodemographic analysis of cases and controls showed an increase incidence T2D in male compared to female patients. The occurrence of T2D was found high in old age patients compared to younger age patients. Comorbidities like hypertension, ischemic heart disease, renal failure, and hypercholestermia occurrence percentage were found much higher in T2D patients than controls. Most of the patients (94%) were found to have family history of diabetes. Diet control and physical activities (exercise) were recorded poor in patients. Similarly, our data shows that almost 100% patients were belonging from low-income families and doing laborious low-paid jobs.

4.1. Study Limitations. Study limitations include limited sample size, and sample collection was restricted only to Khyber Pakhtunkhwa province; our study does not include some intersecting genes that may have strong association with T2D and high percentage of comorbidities in cases. Present study despite of these limitations successfully demonstrated the role of common genetic variants in progression of complex diseases like diabetes in Pakistani cohort. Further, more advance tools of analysis have been used instead of conventional ones.

5. Conclusion

The study demonstrated the association of HLA-B gene with T2D in the study population. The findings suggest strong association of SNP rs2308655 with T2D in Pashtun ethnic population. The other HLA alleles, namely, rs1051488, rs1131500, rs1050341, and rs1131285, were shown to have no/very weak (if any) association with T2D. To our knowledge, this study is first of its kind to report T2D risk variants in patients of Khyber Pakhtunkhwa, Pakistan. To overcome this fatal and costly disease, it is recommended that similar projects should be designed on large scale to screen individuals who are genetically susceptible to T2D and awareness campaigns on genetic and environmental risk factors should

be initiated in general public. This will help reduce/control the prevalence of the disease.

Data Availability

All the needed and necessary information has been provided along with the manuscript. However, the corresponding author can be contacted for any other information related to this paper.

Conflicts of Interest

Authors declare no competing interests.

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Supplementary Materials

Resultant file was filtered for T2D susceptible genes (supplementary file 1). It has three main classes: class I HLAs (A, B, and C), class II HLAs (DQ, DM, DP, DR, and DO), and class III HLAs (CSK2B, SKI2W, C4B, and PBX2) (supplementary file 2). *(Supplementary Materials)*

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Research Article

Changes of Serum Zinc-α2-Glycoprotein Level and Analysis of Its Related Factors in Gestational Diabetes Mellitus

Dongmei Xu,[1] **Jie You**,[2] **Guixia Chen**,[1] **Hongli Su**,[3] **Li Zhang**,[1] **Lingling Cui**,[4] **Zhonglei Li**,[4] **Guoling Huang**,[5] **and Caiying Feng**[2]

[1]*Department of Health, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China*
[2]*Department of Clinical Nutrition, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China*
[3]*Department of Obstetrics and Gynecology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou, Henan 450007, China*
[4]*Department of Nutrition and Food Hygiene, School of Public Health, Zhengzhou University, Zhengzhou Henan 450001, China*
[5]*Department of Financial, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China*

Correspondence should be addressed to Caiying Feng; fcy666666@126.com

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Previous studies have discovered that zinc-α2-glycoprotein (ZAG) is related to insulin resistance and lipid metabolism. The aim of the study is to explore the change of serum ZAG and its related factors in gestational diabetes mellitus (GDM). Eighty newly diagnosed GDM patients were enrolled in the case group, and 80 normal pregnant women were selected as the control group. The differences of baseline data between the two groups were compared, and the change of serum ZAG level and its relationship with related indexes was analyzed. Compared to the control group, the level of serum ZAG in GDM women decreased ($P < 0.001$). What is more, the serum ZAG level of overweight and normal subjects in two groups was also found to have statistical differences. The Pearson correlation (or Spearman correlation) analysis showed that serum ZAG level was negatively correlated with FPG, FINS, HOMA-IR, and TG (all $P < 0.05$) and positively correlated with HDL ($P < 0.05$). Multiple linear regression showed that HDL and HOMA-IR were independent factors of serum ZAG ($P < 0.05$). The level of serum ZAG in patients with gestational diabetes mellitus decreased, and HDL and HOMA-IR are the influencing factors in the case group.

1. Introduction

Gestational diabetes mellitus is one of the common complications of pregnancy. The prevalence of GDM ranges from 1 to 18.5%, which globally has an increasing tendency year by year [1–3]. However, the etiology of GDM has not been clear yet so far, and the pathogenesis needs further study. At present, it is generally believed that maternal obesity and lipid metabolism disorder are related to the occurrence of GDM [4, 5]. Nowadays, many specialists focus their eyes on the role of adipokines, which contributes to the metabolic abnormalities in the mother. A study found statistical difference in adiponectin between GDM patients and normal pregnant women [6]. ZAG is a type of adipocytokine, secreted by adipocytes, which was first isolated and purified from human serum by Burgi et al. in 1961 [7]. It is widely found in human plasma and other various body fluids, with carrier protein, ribonuclease activity, and other functions [8, 9]. In recent years, many studies have shown that ZAG has the function of regulating immunity, cell adhesion, and melanin production [10, 11]. Some studies [12–14] also pointed out that ZAG can be used as a biomarker for the early diagnosis of cancer, and it can participate in regulating tumor cell proliferation and glucose metabolism [15]. It was also found that ZAG is not only involved in the regulation of fat metabolism in obesity but also related to the occurrence of T2DM lipid metabolism disorder [16]. A randomized controlled trial [15] found evidence to suggest that adipose tissue may be a primary source of circulating ZAG in humans and that the downregulation of ZAG expression in adipose tissue

may be responsible for the reduced circulating ZAG levels in T2DM patients. In vivo and in vitro experiments also confirmed that ZAG can promote fat mobilization [17, 18]. Another study showed that ZAG was associated with downregulated lipogenic enzymes and upregulated lipolytic enzyme expressions in adipose tissue [19].

At present, most of previous research is limited to T2DM patients and other related diseases. Compared with the previous study of the relationship between ZAG and GDM, we measured the level of ZAG in the second trimester. Considering the earlier detection time, it may be more meaningful in predicting and controlling GDM. The study's aim is to explore the change of serum ZAG and its related factors in GDM, thus providing a theoretical basis for the metabolism mechanism of GDM patients. And it may provide a new direction for the prevention and treatment of GDM.

2. Materials and Methods

2.1. Subjects. All subjects of gestation age between 24 weeks and 28 weeks were recruited in the study in the Third Affiliated Hospital of Zhengzhou University from July 2018 to June 2019. All the subjects are Chinese. They all were naturally pregnant and had single pregnancy, and they did regular perinatal care and gave birth in the hospital. They undertook 75 g oral glucose tolerance test (OGTT) during their prenatal examination. Newly diagnosed gestational diabetes mellitus patients were selected as the case group. We selected normal pregnant women as controls on the same day when GDM pregnant women were diagnosed by matching the gestational week with that of the GDM pregnant women. What is more, the range of age between the GDM pregnant women and the matching controls is not more than 3 years old. And we excluded the pregnant women with chronic diseases such as diabetes, hypertension, cardiovascular and cerebrovascular diseases, severe liver and kidney diseases, tumors, mental diseases, and other pregnancy complications. Written informed consent was obtained from all participants. The study meets the requirements of the ethics committee, and the study was registered in the Chinese Clinical Trial Registry (ChiCTR2000028811).

2.2. Methods. The demographic and clinical characteristic data were collected, such as height, weight, body mass index (BMI), age, race, residence, systolic pressure (SBP), and diastolic pressure (DBP). Some hematologic and biochemical indexes including fasting blood glucose (FBG), blood glucose, triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol (TC) were also collected.

The enzyme-linked immunosorbent assay (Huamei Bioengineering Co., Ltd., Wuhan, China) was used to detect the level of serum ZAG. The coefficients of variation (CV) for the intra- and interassay were lower than 8% and 10%, respectively.

ELISA kits (Huamei Bioengineering Co., Ltd., Wuhan, China) were used to detect the level of insulin, and the CV for the intra- and interassay were both lower than 15%. The insulin resistance index (HOMA-IR) was calculated and analyzed with homeostasis model assessment [20]. The calculation formula is HOMA-IR = FPG × FINS/22.5.

2.3. Diagnostic Criteria for Gestational Diabetes Mellitus. According to Chinese diagnostic criteria of gestational diabetes mellitus, the critical serum glucose values of fasting and 1 hour and 2 hours after taking glucose were 5.1 mmol/L, 10.0 mmol/L, and 8.5 mmol/L, respectively. Gestational diabetes can be diagnosed if any of the three outcomes is greater than or equal to the critical value in pregnant women with fasting glucose or 75 g OGTT after 24-week gestation.

2.4. Statistical Analysis. The SPSS 21.0 statistical package was used to process the data of the study. Firstly, the 1-sample Kolmogorov-Smirnov test was performed to verify the normal distribution of the quantitative variables. And then normally distributed data were expressed as mean ± SD, while the quantitative data of abnormal distribution were expressed as median (25-75th percentile). And qualitative data were expressed as a ratio (or percentage). The independent samples t-tests, Mann-Whitney U test, or chi-square test was used to explore the difference between the two groups. The Pearson (or Spearman) correlation analysis and multiple linear regression analysis were used to evaluate the association between the indicators. Two-tailed P values less than 0.05 were regarded as statistically significant.

3. Results

3.1. Comparison of Baseline Clinical and Metabolic Characteristics between the Two Groups. According to the inclusion criteria and exclusion criteria, a total of 160 subjects were enrolled in the study, with 80 subjects in each group. The age of the case group and the control group were 30.08 ± 3.43 years and 30.50 ± 3.88 years, respectively. No significant differences were found between the two groups ($P > 0.05$). As expected, there were higher levels of fasting glucose, gain weight, HbA1c, and fasting insulin in the case group, and the difference between the two groups was statistically significant ($P < 0.05$). HOMA-IR in the case group has higher level than that in the control group, and the difference was statistically significant ($P < 0.001$). We also investigated the level of serum lipid in the two groups, and the results showed that the case group has higher level of TG and lower level of HDL, and the difference was significant ($P < 0.05$). However, there was no statistical difference in TC and LDL between the two groups ($P > 0.05$). The other baseline clinical and metabolic characteristics such as height, SBP, and SDP showed no statistical difference in the two groups ($P > 0.05$), as shown in Table 1.

3.2. Comparison of Serum ZAG Levels between the Case Group and Control Group. Compared to the control group, the serum ZAG level in the case group was decreased, and the difference was statistically significant (43.94 ± 14.51 mg/L vs. 62.57 ± 19.05 mg/L, $P < 0.001$, Figure 1). With BMI = 25.0 kg /m^2 as the cut-off point, we divided the case group and the control group into the normal group (BMI < 25.0 kg/m^2) and overweight group (BMI ≥ 25.0 kg/m^2). The ZAG levels of the

Table 1: The clinical and metabolic characteristics of the two groups.

Characteristics	Case group ($N = 80$)	Control group ($N = 80$)	t/Z	P
Age (years)	30.08 ± 3.43	30.50 ± 3.88	-0.734	0.464
Height (cm)	161.86 ± 4.71	161.35 ± 4.40	-0.740	0.460
Weight (kg)	62.73 ± 10.65	58.18 ± 7.18	-3.168	0.002
SBP (mmHg)	114.21 ± 6.90	113.26 ± 5.41	-0.970	0.334
DBP (mmHg)	68.16 ± 7.36	67.50 ± 5.84	-0.621	0.535
BMI (kg/m^2)	24.58 ± 3.93	22.96 ± 2.82	-3.112	0.002
Gain weight (kg)	6.91 ± 2.14	5.32 ± 1.23	-2.474	0.015
HbA1c (%)	5.74 ± 0.45	4.81 ± 0.33	-14.906	<0.001
Fat mass (kg)	20.8 (16.93, 22.52)*	20.3 (15.35, 21.98)*	-2.273	0.023
Fat (%)	34.47 ± 6.01	33.23 ± 4.63	-1.521	0.130
FPG (mmol/L)	5.03 ± 0.60	4.45 ± 0.35	-7.817	<0.001
OGTT-1 h	9.49 ± 1.68	6.92 ± 1.35	-10.723	<0.001
OGTT-2 h	8.44 ± 1.55	6.40 ± 1.00	-9.907	<0.001
FINS (mU/L)	7.56 (5.69, 14.4)*	4.92 (4.01, 10.44)*	-4.363	<0.001
HOMA-IR	1.59 (1.28, 3.22)*	0.94 (0.79, 1.81)*	-5.453	<0.001
TC (mmol/L)	5.51 ± 1.32	5.59 ± 1.87	0.293	0.770
TG (mmol/L)	2.83 (2.21, 3.48)*	2.58 (1.89, 3.36)*	-7.152	<0.001
HDL (mmol/L)	1.73 ± 0.47	2.07 ± 0.62	3.943	<0.001
LDL (mmol/L)	3.06 ± 0.69	2.86 ± 0.86	-1.641	0.103
Residence			1.604	0.205
City, n (%)	48 (60.00)	55 (68.75)		
Rural, n (%)	32 (40.00)	25 (31.25)		

SBP: systolic pressure; DBP: diastolic pressure; BMI: body mass index; HbA1c: glycosylated hemoglobin; fat (%): percent of body fat; FPG: fasting blood glucose; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TC: total cholesterol; ZAG: zinc-α2-glycoprotein. *Value data are presented as median (25-75th percentile), and others are presented as mean ± SD.

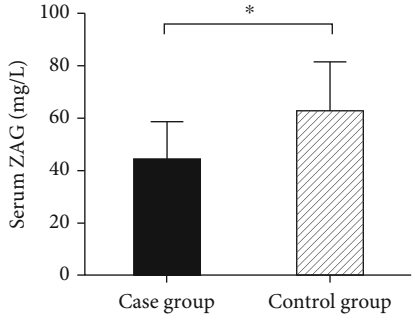

Figure 1: Comparison of serum ZAG between the two groups.

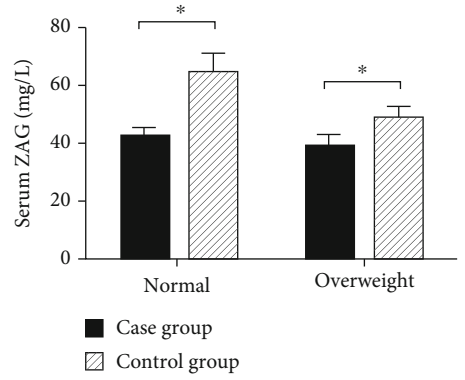

Figure 2: Comparison of serum ZAG levels between the overweight and normal groups in the case group and control group.

normal group were 41.23 ± 2.15 mg/L in the case group and 64.06 ± 4.27 mg/L in the control group, while the ZAG levels of the overweight group were 39.83 ± 3.08 mg/L in the case group and 48.69 ± 2.97 mg/L in the control group. We found that the level of serum ZAG was declined in both the overweight group and the normal group, and the differences were statistically significant ($P < 0.05$), as shown in Figure 2.

3.3. Correlation Analysis of Serum ZAG and Related Factors in the Case Group. With ZAG as the dependent variable and other clinical indicators as independent variables, the correlation analysis was conducted. The results are shown in Table 2. It showed that serum ZAG levels of pregnant women in the case group were negatively correlated with FPG, FINS, HOMA-IR, and TG ($r = -0.416$, -0.167, -0.236, and -0.328, $P < 0.05$). And the results also showed that serum ZAG was positively correlated with HDL ($r = 0.279$,

Table 2: Correlation between serum ZAG and related indexes in the case group.

	Age	BMI	Fat mass	Fat (%)	FPG	FINS	HOMA-IR	TC	TG	HDL	LDL
r	0.089	-0.219	-0.015	-0.086	-0.416	-0.167	-0.236	0.018	-0.328	0.279	0.153
P	0.432	0.051	0.895	0.450	<0.001	0.035	0.015	0.876	<0.001	0.012	0.174

$P = 0.012$). However, no relationship was found between the serum ZAG and age, BMI, fat mass, etc. ($P > 0.05$).

3.4. Multiple Linear Regression Analysis of Serum ZAG in the Case Group. FPG, FINS, HOMA-IR, TG, and HDL were included in the regression model as independent variables, and the results showed that HOMA-IR ($\beta = -3.168$, $P < 0.05$) and HDL ($\beta = 2.551$, $P < 0.05$) were independent influencing factors of serum ZAG. When conducting the multiple linear regression analysis, stepwise regression was used. What is more, the inclusion criterion was $P \leq 0.05$ and the exclusion criterion was $P \geq 0.1$.

4. Discussion

According to the study, we found that the level of serum ZAG level decreased in GDM patients, compared to the control group. And subgroup analysis showed that the GDM patients also tended to have lower ZAG levels in both overweight pregnant woman and normal subjects. Finally, the regression analysis showed that HOMA-IR and HDL were independent influencing factors of serum ZAG level in GDM patients. But whether serum ZAG has a certain role in the metabolism of serum glucose and lipid in GDM patients still needs further study. Some experts have done some research. Yang et al. [15] found that ZAG was related to insulin resistance, which was consistent with the view. Naf et al. [6] also pointed out that serum ZAG level in GDM patients was related to HDL, suggesting that ZAG was involved in lipid metabolism in GDM patients. However, no statistical difference was found between the GDM group and the NGT group in serum ZAG level, which may be caused by factors such as different races, research methods, and sample size. In addition, Naf et al. measured the serum ZAG level before delivery, while our study collected the detection indicators of pregnant women with GDM at 24 to 28 weeks, which may lead to the difference between the two studies.

In summary, the lower levels of serum ZAG might relate to HOMA-IR and HDL. Serum ZAG might play a certain role in lipid metabolism and insulin resistance in patients with GDM. Some experts found that ZAG could reduce adiponectin, insulin receptor substrate-1, and glucosetransporter-4 gene expression in primary human adipocytes, which indicates that ZAG might play an important role in modulating whole-body and adipose tissue insulin sensitivity [21]. Currently, there are few studies on the relationship between serum ZAG and GDM, and more evidence-based medical evidence is still needed. The potential limitations of our study are the relatively small sample and the geographical distribution. Therefore, a multicenter and multiarea large-sample prospective study is still needed to further clarify the relationship between serum ZAG and GDM.

Data Availability

The figures and tables used to support the findings of this study are included within the article.

Disclosure

We have submitted the manuscript in the preprint.

Conflicts of Interest

There is no conflict of interest to declare.

Authors' Contributions

C.Y.F., J.Y., and L.L.C. conceived and designed the study. G.X.C., H.L.S., L.Z., and Z.L.L. conducted the study and the data collection. G.L.H. made the statistical analysis. D.M.X. and J.Y. drafted and revised the manuscript. Finally, D.M.X. wrote the article. All authors have read and approved the final manuscript.

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Research Article

Correlation Analysis between GDM and Gut Microbial Composition in Late Pregnancy

Genxia Li,[1] **Pan Yin**,[1] **Shuhui Chu**,[1] **Wanli Gao**,[1] **Shihong Cui**,[1] **Shuhua Guo**,[1] **Yajuan Xu**,[1] **Enwu Yuan**,[1] **Texuan Zhu**,[2] **Jie You**,[3] **Junya Zhang**,[1] **and Manman Yang**[1]

[1]*Obstetrics Department, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China*
[2]*Laboratory Department, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China*
[3]*Nutrition Department, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China*

Correspondence should be addressed to Genxia Li; ligenxia@163.com

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The prevalence of GDM is very high worldwide. The specific pathogenesis of GDM is currently not very clear. Recent research suggests that changes in the intestinal flora during pregnancy play a key role in it. Therefore, this study is aimed at exploring the characteristics of the intestinal flora of patients with gestational diabetes in the third trimester of pregnancy and at finding the intestinal flora with significant differences in healthy pregnant women to provide a basis for future clinical attempts of using intestinal microecological agents to treat gestational diabetes mellitus (GDM). We sequenced the V3-V4 regions of the 16S ribosomal ribonucleic acid (rRNA) gene from stool samples of 52 singleton pregnant women at >28 weeks of gestation. Our results showed that there were significant differences between the NOR group vs. GDM group and the G group vs. LG group among *Bacteroides*, *Firmicutes*, and *Firmicutes*/*Bacteroides*. At the species level, there were significant differences in the abundance of eight species in the NOR and GDM groups. Among them, the relative abundance of *Clostridium_spiroforme*, *Eubacterium_dolichum*, and *Ruminococcus_gnavus* was positively correlated with FBG, and *Pyramidobacter_piscolens* was negatively correlated with FBG, whereas there were significant differences in the abundance of five species in the G and LG groups. Functional analysis showed that there were differences in the biosynthesis and metabolism of polysaccharides, digestive system, classification, and degradation of the intestinal microbes between the NOR and GDM groups and between the G and LG groups. These results indicated that the gut microbes between GDM patients in the third trimester of pregnancy and healthy controls had essential characteristic changes and might be involved in the regulation of patients' blood glucose levels.

1. Introduction

GDM refers to the first occurrence of varying degrees of glucose metabolism abnormalities during pregnancy. It is a common complication of pregnancy [1, 2]. According to the International Diabetes Federation (IDF), one in six mothers (16.8%) of live births had some form of hyperglycemia during pregnancy, and 84% of them were diagnosed with GDM [3]. The incidence of GDM in Asian populations could be up to 20%. Although most GDM patients return to normal postpartum glucose metabolism, 20% of them continue to have impaired glucose tolerance or impaired fasting blood glucose (FBG) postpartum, and GDM patients have a significantly higher lifetime risk of type 2 diabetes, which is 7.4 times that of non-GDM patients [4]. Recently conducted research also found that independent of obesity or type 2 diabetes, GDM is also a risk factor for hypertension, metabolic syndrome, and cardiovascular disease [5–8]. These aspects not only have a serious impact on the short- and long-term health of mothers and babies but also continue to increase medical costs and significantly increase the social burden. Therefore, early diagnosis of GDM or early identification of high-risk populations is particularly important.

The specific pathogenesis of GDM is currently not very clear. Recent research suggests that changes in the intestinal flora during pregnancy play a key role in it [9, 10]. In 2007, the "Human Microbiome Project" was proposed. In recent years, with the emergence of metagenomics and

metabolomics, more and more diseases have been demonstrated to be related to the intestinal flora [11–13]. The normal intestinal flora and the mucosal surface form a natural barrier, which plays an essential role in digestion and absorption, regulating immune function and preventing the invasion of pathogenic bacteria. Studies at home and abroad have shown that intestinal microflora disorders play a key role in the pathogenesis of obesity, type 2 diabetes, inflammatory bowel disease, liver disease, kidney injury, autism, and other diseases [14–18]. Pregnancy, especially in the third trimester, is a unique period. As the body's metabolic needs increase, the intestinal flora, as a "metabolic organ," will also undergo adaptive changes [19, 20]. The characteristics of the intestinal flora in the third trimester of pregnancy and its correlation with the onset of GDM have become research hotspots.

In this study, we used 16S rRNA high-throughput sequencing technology to detect the intestinal bacteria of pregnant women with GDM and normal pregnant women at different stages of pregnancy (28–36 weeks and 36–41 weeks) and analyzed the different flora of the three groups of pregnant women. If a difference exists between the intestinal flora of normal pregnant women and that of patients with GDM, this would help us understand the pathogenesis of GDM and provide a theoretical basis for future treatment of gestational diabetes by supplementing probiotics. Therefore, the aim of this study was to investigate whether there are differences between the intestinal flora of normal pregnant women and those with GDM by using 16S rRNA high-throughput sequencing technology to determine the intestinal flora of normal pregnant women and those with GDM in the third trimester of pregnancy.

2. Materials and Methods

2.1. Study Population. From October 1, 2018, to December 30, 2019, 52 singleton pregnant women at more than 28 gestational weeks were recruited from the outpatient department of the Third Affiliated Hospital of Zhengzhou University. Of these, 23 had GDM and were further divided into a longer gestational week (LG group, $n = 12$) and shorter gestational week (G group, $n = 11$) based on whether or not the gestational age was greater than 36 weeks. The remaining 29 were normoglycemic pregnant women (NOR group). The inclusion criteria were as follows: pregnant women without prepregnancy diabetes, hypertension, serious damage to other organs, other metabolic diseases, other endocrine diseases, history of chronic diseases of the digestive tract, diarrhea, or other gastrointestinal diseases in the past 4 weeks; those who were taking antibiotics or intestinal probiotics in the past 4 weeks; and those with abnormal routine stool examination. We excluded those specimens with insufficient amounts of specimen retention or contamination during specimen conservation. The Medical Ethics Committee of the Third Affiliated Hospital of Zhengzhou University approved the present study, and all participants provided a written informed consent form.

2.2. Diagnostic Criteria for GDM. The diagnosis of GDM was made if any of the following criteria were met: (1) FBG $\geq$ 5.1 mmol/L, (2) 1 h blood glucose post 75 g oral glucose load $\geq$ 10.0 mmol/L, and (3) 2 blood glucose post 75 g oral glucose load $\geq$ 8.5 mmol/L [21].

2.3. Sample Collection. Stool samples (1 g) were collected before breakfast and put into sterile plastic tubes. Adopting a sampling box to keep samples at low temperatures during 0.5 h transport back to the lab, in a −80°C refrigerated storage, completes DNA extraction within 48 h.

2.4. Detection of Biochemical Indicators. We used the Hitachi 7600-020 automatic biochemical analyzer to detect FBG (hexokinase method detection), total cholesterol (enzyme colorimetric detection), triacylglycerol (TG, enzyme colorimetry detection), high-density lipoprotein cholesterol (catalase removal method detection), and low-density lipoprotein cholesterol (catalase removal method detection). The five test item reagents were all produced by Sichuan Mike Biological Technology Co. Ltd. All experimental processes were tested with high- and low-level quality control products to ensure the accuracy of test results.

2.5. Bacterial DNA Extraction and 16S rRNA Gene Sequencing. DNA extraction and 16S rRNA gene sequencing were conducted by the Wuhan Huada Gene Sequencing Center. The extraction of total bacterial DNA from stool samples was conducted according to the instructions of the BGI Stool Genome Extraction Kit. A microspectrophotometer was used to detect the concentration and purity of DNA. When the ratio of absorbance 260/280 is 1.7 to 1.9, the extracted DNA is considered usable. We designed 16S rRNA gene PCR primers using total DNA as a template to amplify the 16S rRNA V3-V4 hypervariable region. Subsequently, the Illumina Hiseq 2500 PE250 protocol was used for targeted amplicon sequencing. We used the Flash software to merge the original opposite sequences and divide them with tags. The minimum overlap was 15 bp, and the mismatch rate was <0.1.

2.6. OTU Clustering. The operational taxonomic unit (OTU) refers to a unified mark set artificially for a certain taxonomic unit (line, genus, species, grouping, etc.) to facilitate analysis in phylogeny or population genetics research. We used the software USEARCH (v7.0.1090) to cluster the spliced tags into OTUs and usually cluster tags with a similarity of more than 97% into one OTU. The abundance of OTU preliminarily illustrates the species richness of the sample.

2.7. OTU Species Annotation. After obtaining the representative sequence of OTU, we used RDP classifier (v2.2) software to compare the representative sequence of OTU with the Greengenes database for species annotation and set the confidence level at 0.8. The annotation results were filtered as follows: (1) remove OTUs without annotation results and (2) remove annotation results that do not belong to the species in the analysis project. For example, if the sample is made of 16S bacteria and OTU annotates Archaea, it will be removed.

2.8. Statistical Processing. We used software R (v3.1.1)'s VennDiagram package to generate Venn diagrams and OTU Core-Pan diagrams and R (v3.2.1)'s mixOmics package for OTU PLS-DA analysis. We used R (v3.1.1)'s gplots package to generate a species abundance heat map whose distance algorithm was Euclidean and whose clustering method was complete. GraPhlAn (https://huttenhower.sph.harvard.edu/graphlan) was used to generate a species composition map. R (v3.4.1)'s cluster and clusterSim packages were used for flora typing analysis. R (v3.2.1) was used to generate an alpha diversity box plot. R (v3.4.1)'s ggplot package was used to generate Beta diversity index box plots. LEfSe (https://huttenhower.sph.harvard.edu/galaxy/) was used to analyze the cluster diagram and the LDA diagram. We used R (3.4.1) and the Kruskal-Wallis test to screen the different species and used R (v3.4.1) and Picrust software to predict the function of the flora. Cytoscape was used to draw species network diagrams. SPSS 23.0 was used for statistical processing. Normally distributed measurement data were represented by $x \pm s$, and count data were represented by "percentage (%) or rate." The comparison between the two groups was conducted by a t-test. Pearson's correlation coefficient was used to assess the correlation between blood glucose and different flora. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of General Conditions and Biochemical Indicators between the Two Groups. According to the statistical analysis of the collected general data and laboratory data, there was no significant difference in age and gestational age between the GDM and the NOR groups. The levels of BMI, FBG, 2 h blood glucose, HbA1c and TG, CHOL, and LDL in the GDM group were significantly higher than those in the NOR group, and the HDL level was significantly lower in the GDM group than in the NOR group ($P < 0.01$). The comparison between the G and LG groups showed that only the difference in gestational age between the two groups was significant ($P < 0.01$), and differences in other indicators were not significant. Details are found in Table 1.

3.2. OTU Sequence Diversity and Richness. After 52 samples were sequenced, a total of 866 OTUs were generated after clustering. The sequence values obtained after OTU clustering were analyzed, and the results were as follows. The average effective sequence obtained from the fecal flora of the NOR group was 74841, and 779 OTUs were obtained after merging with 97% similarity, and the sequencing coverage depth (coverage index) was 0.99932. The average effective sequence obtained from the fecal flora of the GDM group was 74846. After 97% similarity merging, 721 OTUs were obtained, and the sequencing coverage index was 0.99938. Additionally, the GDM group was classified as the G group when the pregnancy was less than 36 weeks. After 97% similarity, 608 OTUs were obtained, and the sequencing coverage index was 0.99934. The LG group was made of pregnant women at ≥36 weeks of pregnancy, and 563 OTUs were obtained after 97% similarity merging, and the sequencing coverage index was 0.99941. The sequencing depth was above 0.99, indicating that the probability of undetected sequences in the sample group was low.

The analysis of the alpha diversity index of the two groups of samples at the 97% similarity level showed that the observed species index, Chao index, Ace index, and coverage index levels of the GDM and NOR groups were similar, and there was no significant difference. The Shannon index of the GDM group was significantly higher than that of the NOR group, whereas the Simpson index was lower than that of the NOR group. It can be roughly estimated that the α diversity of the GDM group is greater than that of the NOR group. Additionally, among pregnant women with GDM, the fecal flora of gestation < 36 weeks (G group) and gestation ≥ 36 weeks (LG group) were analyzed by the alpha diversity index at the 97% similarity level. The average values of Chao and Ace indexes in the G group were 235 and 239, respectively, and in the LG group were 233 and 237. The results showed that the abundance of bacteria in the G group was higher than that in the LG group, but the difference was not statistically significant ($P \geq 0.05$). The mean values of the Shannon and Simpson indices in group G were 3.21 and 0.10, respectively, and the mean values in the NOR group were 2.75 and 0.19, respectively. The diversity of flora in the G group was higher than that in the LG group, but the difference was not statistically significant ($P \geq 0.05$). The specific results are shown in Figure 1.

A Venn diagram analysis showed that the NOR and GDM groups shared 634 OTUs (Figure 2(a)). PLS-DA analysis showed that the NOR and GDM groups were clustered and distinguished well (Figure 2(b)). It suggested that there were also significant differences in the composition and structure of the sample flora between the two groups.

To obtain the species classification information corresponding to each OTU, we used the RDP classifier Bayes algorithm to conduct taxonomic analysis on the OTU representative sequence and counted the bacterial composition of each sample at the level of phylum, class, order, family, genus, and species.

The heat map cluster analysis can visually display the clustering of samples from the same treatment or similar environment and reflect the similarity and difference in the bacterial composition of the samples (Figures 3(a) and 3(c)). The GraPhlan species composition map mainly displays the overall visual display of the species composition of each taxonomic level of the sample, distinguishes each taxum with different colors, reflects the species abundance of each taxonomy level through the size of the node, and uses the color depth of the outer ring heat map to represent the species abundance of each group. It can be used to discover dominant microbial groups (Figures 3(b) and 3(d)).

We analyzed the relative abundance of species in the GDM and NOR groups at the phylum level. In the NOR group, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the predominant flora, accounting for 68.2%, 23.2%, and 7.4%, respectively, of the total flora, whereas in the GDM group, accounting for 53.6%, 38.1%, and 5.1% of the total flora. The results showed that the proportion of *Bacteroides* in the GDM group was significantly lower than that in the NOR group, and the proportion of *Firmicutes* in the GDM

Table 1: Comparison of general conditions and biochemical indicators between the two groups of pregnant women ($x \pm s$).

	NOR (n, 29)	GDM (n, 23)	G (n, 11)	LG (n, 12)
Age (years)	29.00 ± 1.88	29.80 ± 2.19	29.64 ± 2.29	29.67 ± 2.19
Gestational week (weeks)	36.10 ± 4.03	35.26 ± 3.65	32.03 ± 2.56	38.23 ± 1.35*
Prepregnancy BMI (kg/m^2)	21.39 ± 1.37	23.64 ± 1.36*	23.62 ± 1.39	23.65 ± 1.39
FBG (mmol/L)	4.44 ± 0.42	5.29 ± 0.58*	5.22 ± 0.76	5.36 ± 0.38
2 h blood glucose (mmol/L)	6.60 ± 0.73	9.30 ± 1.11*	9.23 ± 1.31	9.37 ± 0.94
HbA1c (%)	5.05 ± 0.46	5.48 ± 0.21*	5.53 ± 0.22	5.44 ± 0.20
TG (mmol/L)	2.11 ± 0.687	3.09 ± 1.20*	3.36 ± 1.36	2.85 ± 1.03
CHOL (mmol/L)	4.96 ± 0.56	5.78 ± 0.79*	5.86 ± 0.76	5.70 ± 0.85
HDL (mmol/L)	2.10 ± 0.34	1.81 ± 0.29*	1.73 ± 0.25	1.88 ± 0.32
LDL (mmol/L)	2.69 ± 0.36	3.07 ± 0.54*	3.10 ± 0.64	3.04 ± 0.47

*$P < 0.05$.

group was significantly higher than that in the NOR group. The ratio of *Firmicutes*/*Bacteroides* in the GDM group (0.71) was significantly higher than that of the NOR group (0.34).

The dominant bacteria in the G and LG groups were Bacteroidetes (43.79% and 62.15%) and Firmicutes (48.29% and 29.19%). Bacteroidetes were significantly higher compared with the G group, and Firmicutes were significantly lower compared with the G group. The Firmicutes/Bacteroidetes ratio (1.10) of the G group was significantly higher than that of the LG group (0.47).

3.3. Analysis of Species Differences in Intestinal Microbiota. The LEfSe cluster analysis of the GDM group and the NOR group was conducted by LDA, and the results are shown in Figures 4(a) and 4(b). In the NOR group, nine groups of bacteria affected the difference between the groups, namely, *Bacteroidetes*, *Bacteroidales*, *Bacteroidia*, *Betaproteobacteria*, *Alcaligenaceae*, *Sutterella*, *Burkholderiales*, *Pyramidobacter*, and *Dethiosulfovibrionacea*. In the GDM group, the groups of bacteria that affected the difference between the groups were *Coriobacteriaceae*, *Coriobacteriia*, *Coriobacteriales*, *Collinsella*, *Dorea*, *Coprococcus*, *Ruminococcus*, *Ruminococcaceae*, *Lachnospira*, *Blautia*, *Lachnospiraceae*, *Clostridiales*, *Clostridia*, and *Firmicutes*.

Furthermore, through the Wilcoxon rank-sum test and the Mann-Whitney U test, the species of the two groups of samples in the phylum, class, order, family, genus, and species level for significant difference analysis and the difference in results at the species level are shown in Figures 4(a) and 4(b) and Table 2. The relative abundance of *Blautia producta*, *Clostridium spiroforme*, *Collinsella aerofaciens*, *Coprococcus catus*, *Eubacterium dolichum*, *Pyramidobacter piscolens*, *Ruminococcus callidus*, *Ruminococcus gnavus*, etc., differed significantly between the NOR and GDM groups. The above differences were statistically significant ($P < 0.05$).

3.4. Correlation Analysis of Intestinal Microbes and Blood Sugar. To understand the close relationship between intestinal bacteria and blood glucose metabolism, the correlation between the relative abundance of the abovementioned different bacterial species and FBG was analyzed separately. The results are shown in Figure 5. The relative abundance of *Clostridium spiroforme* ($r = 0.3284$, $P = 0.0175$), *Eubacterium dolichum* ($r = 0.3333$, $P = 0.0158$), and *Ruminococcus gnavus* ($r = 0.3573$, $P = 0.0093$) in the NOR and GDM groups was positively correlated with FBG. *Pyramidobacter piscolens* ($r = -0.3497$, $P = 0.0111$) was negatively correlated with FBG. Other bacteria had no correlation with FBG.

3.5. Analysis of Differences in Intestinal Microbial Function. We calculated the abundance of each functional category based on the information in the KEGG database and the OTU abundance information. Additionally, for pathway, we used PICRUSt to obtain three levels of metabolic pathway information and also to obtain the abundance table of each level. Simultaneously, the 16S species information was mapped with the functional gene composition in the COG database to obtain the function prediction results. The COG database had two levels, namely, denoted cog_level1 and cog_level2.

After we predicted the functions of all samples, we used the Wilcoxon test to find the difference function between each group. The comparison results of KEGG level2 and COG level2 of the NOR group vs. the GDM group and the G group vs. the LG group are shown in Figure 6. The results of the difference from the comparison of cog_level2 showed that the intestinal microbes of the NOR and GDM groups were significantly different in terms of cell wall/membrane/envelope biogenesis, organic ion transport and metabolism, posttranslational modification, protein turnover, and chaperones, transcription, function unknown, intracellular trafficking, secretion, and vesicular transport (Figure 6(a)). The gut microbes in the G and LG groups had significant differences in amino acid transport and metabolism, replication, recombination and repair, cell wall/membrane/envelope biogenesis, and transcription (Figure 6(b)). The kegg_level2 difference comparison results showed that the intestinal microbes of the NOR and GDM groups were significantly different in terms of poly characterized, transcription, glycan biosynthesis and metabolism, transport and catabolism, digestive system, membrane transport, infectious diseases, folding, sorting and

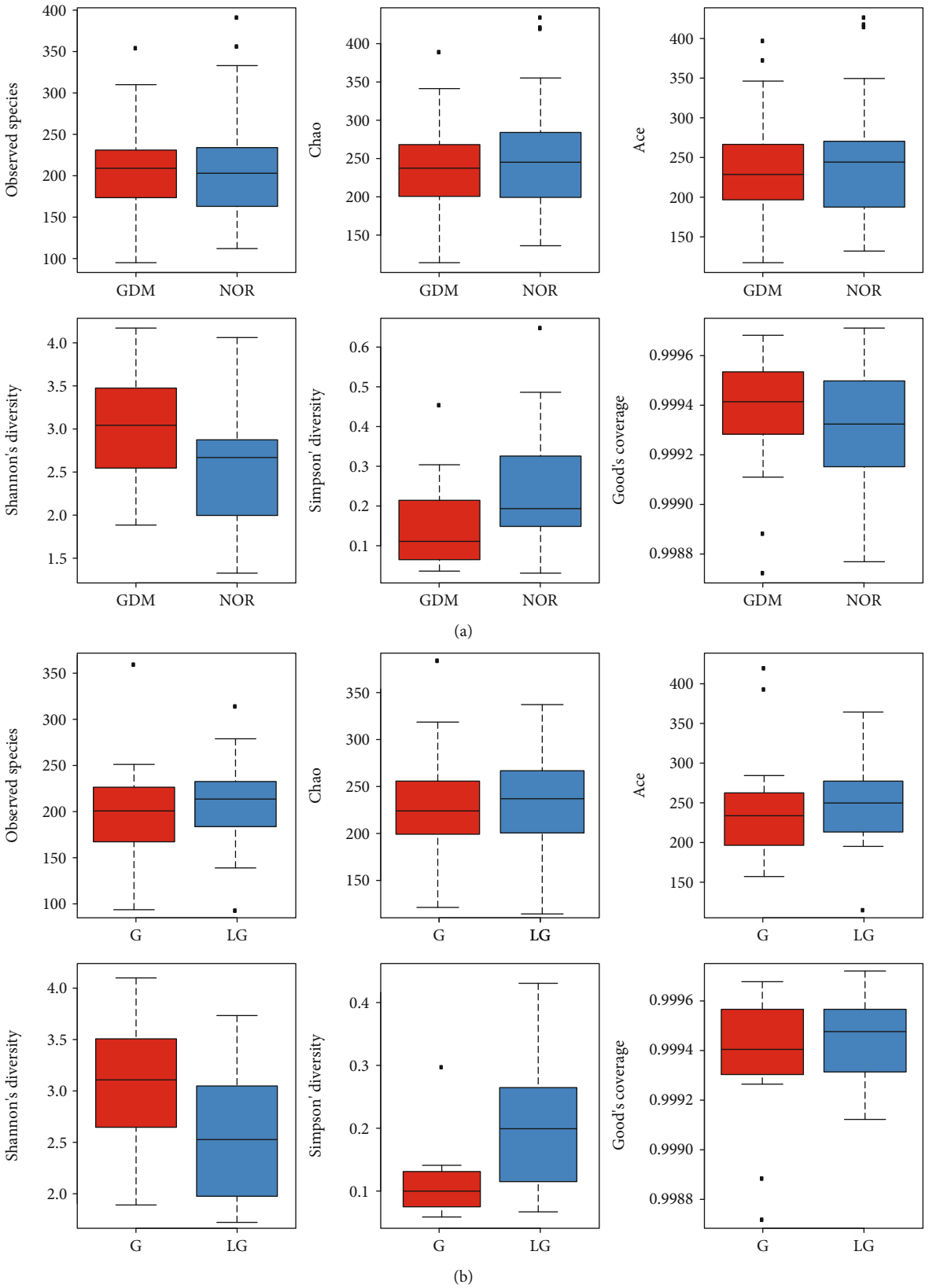


Figure 1: Analysis of OTU α diversity of intestinal flora in the NOR and GDM groups and the G and LG groups. (a) Comparison of the results of OTU α diversity analysis of intestinal microbes in the NOR and GDM groups. (b) Comparison of the results of OTU α diversity analysis of intestinal microbes in the G and LG groups.

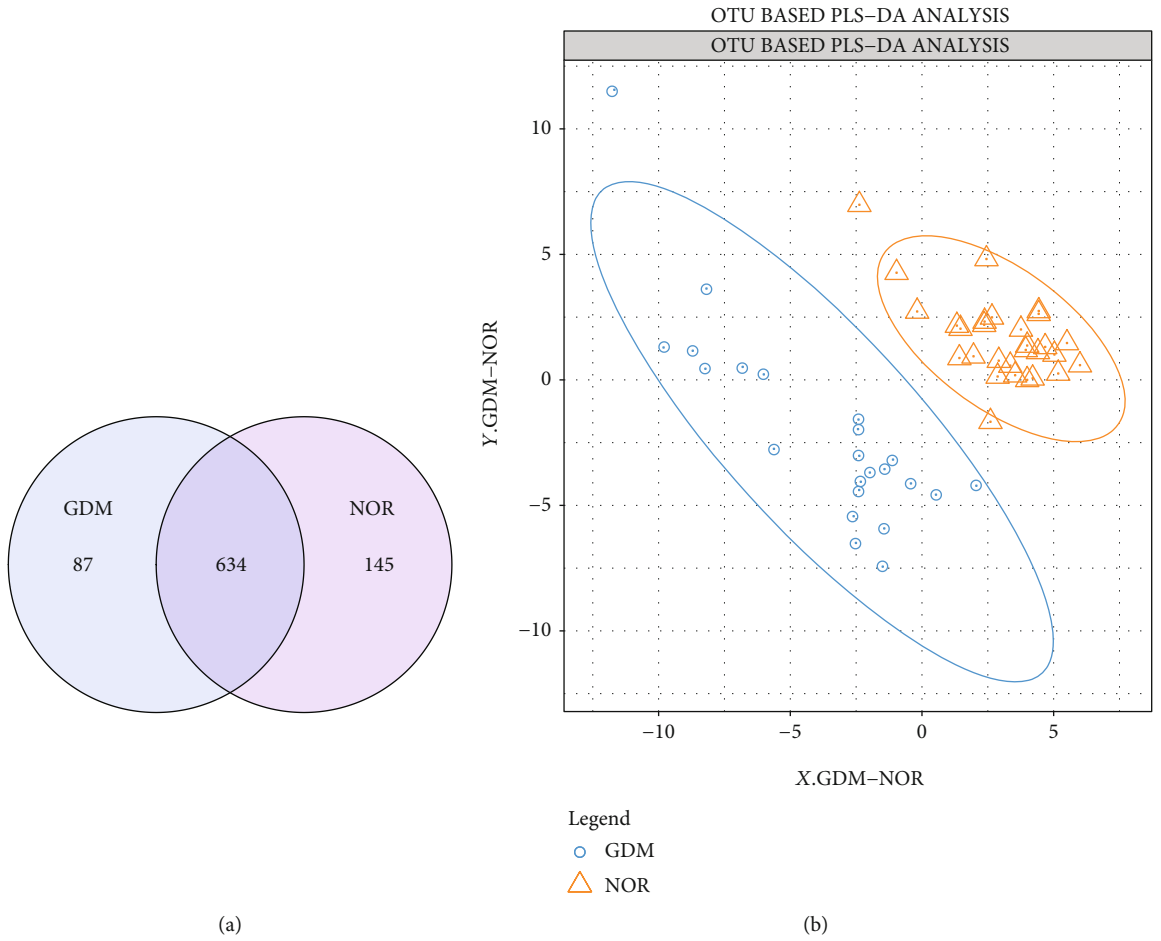


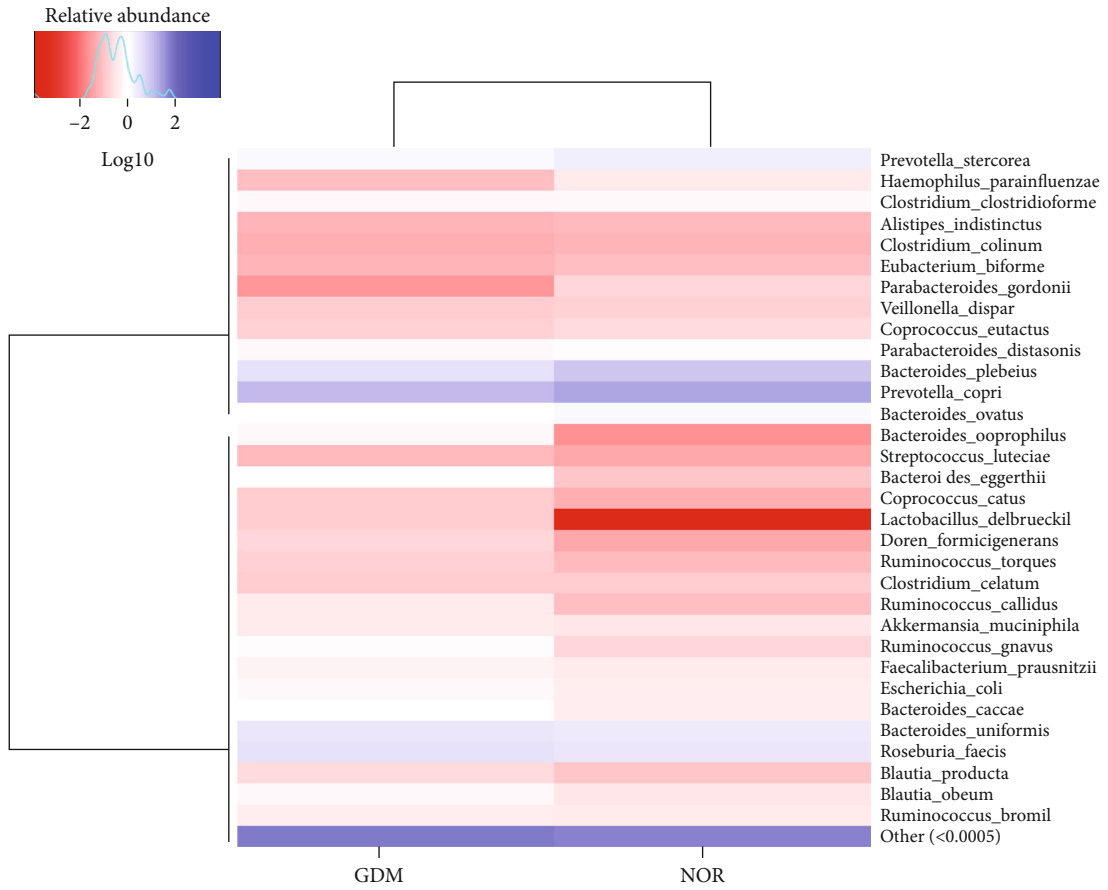
Figure 2: Comparison of the intestinal flora composition of the NOR and GDM groups. (a) Venn diagram showing the overlap of OTUs. (b) PLS-DA analysis of the difference in intestinal flora between the NOR and GDM groups.

degradation, cellular processes and signaling, nucleotide metabolism, and others (Figure 6(c)). The intestinal microbes of the G group and LG group were significantly different in terms of the digestive system, glycan biosynthesis and metabolism, infectious diseases, nucleotide metabolism, and metabolism of terpenoids and polyketides (Figure 6(d)).

4. Discussion

As a common metabolic disease in pregnant women, GDM seriously endangers the life, health, and safety of mothers and their offspring [22]. Studies have pointed out that changes in the intestinal flora might be related to the pathogenesis of GDM [23]. More and more scholars have noticed that changes in the structure of the intestinal flora could be the culprit in many metabolic diseases, such as obesity and type 2 diabetes, and gestational diabetes as a form of diabetes. In recent years, there has been more and more evidence that its onset could be related to the structural changes of the intestinal flora [24, 25]. Studies in China have found that compared with healthy pregnant women (mainly 24–28 weeks of gestation), the alpha diversity of gut microbiota of pregnant women with GDM of the same gestational age is reduced [26]. However, some studies have shown that the abundance of intestinal flora in pregnant women with GDM in the second trimester is higher than that of healthy pregnant women, but there is no significant difference in alpha diversity between the two groups [27]. This study showed that the alpha diversity of the intestinal flora of pregnant women with GDM in the third trimester was significantly higher than that of healthy pregnant women of the same gestational age. The difference between the three studies cannot be underlooked based on the gestational age. This shows that the current research on intestinal flora is still quite different, which might be related to factors such as differences in races, ages, dietary structure, living habits, experiments, and statistical methods. Generally, the study of intestinal flora and GDM lacks prospective studies with large samples and multiple regions, and scholars still need to continue exploring.

Additionally, the analysis of the relative abundance of species in the GDM and NOR groups showed that the dominant bacterial groups were *Bacteroidetes* and *Firmicutes*, but the proportion of *Bacteroidetes* in the GDM group was significantly lower than that in the NOR group, and the proportion of *Firmicutes* was significantly higher than that in the NOR group. The *Firmicutes*/*Bacteroidetes* ratio (0.71) of the GDM group was significantly higher than that of the NOR



(a)

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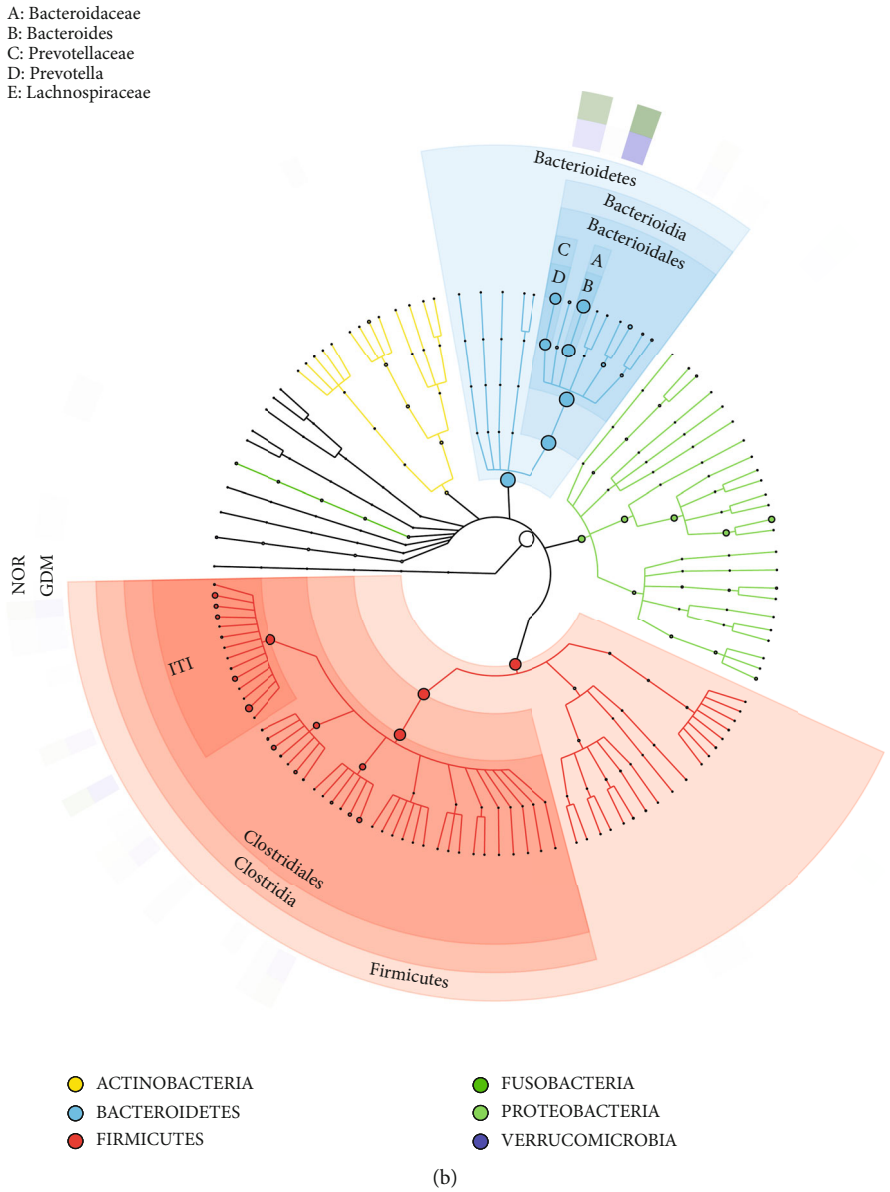

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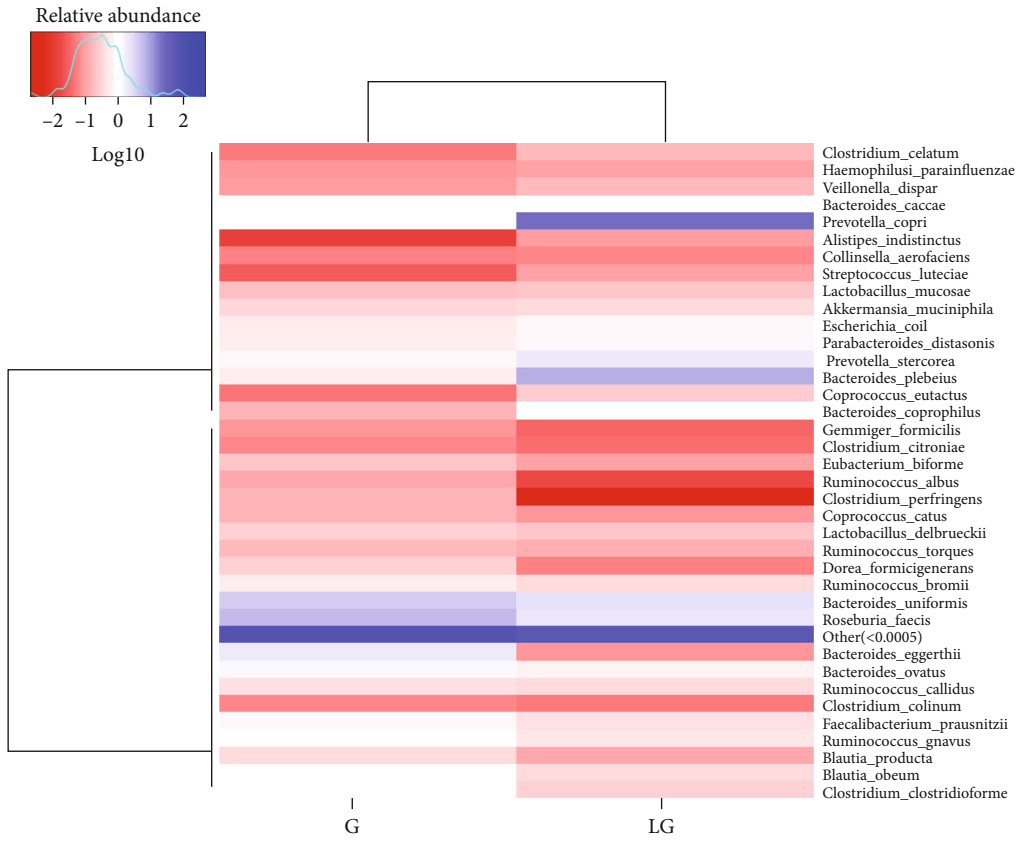

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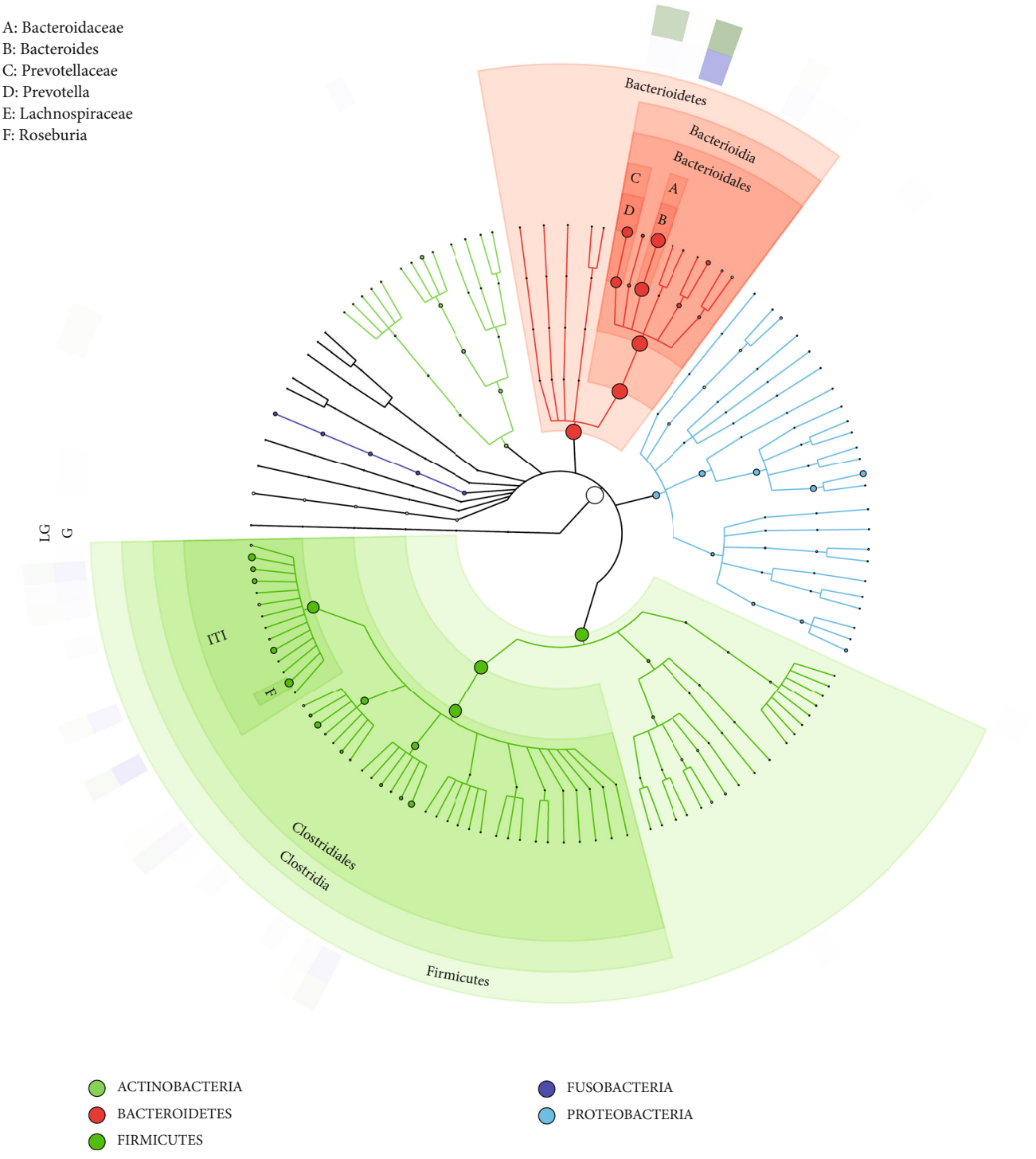


(d)

Figure 3: OTU species composition analysis. (a, c) NOR group, GDM group, G group, and LG group heat map cluster analysis. (b, d) NOR group, GDM group, G group, and LG group GraPhlan species composition analysis.

group (0.34). *Firmicutes* and *Bacteroidetes* are the two main dominant bacterial groups in the intestines. They can maintain the energy balance of the host by participating in the metabolism of fat and bile acids. Their ratio (*F*/*B* value) is often used as an indicator of the composition of the intestinal flora of different individuals [28–31]. A scholar named Ferrocino found that from the second to third trimesters of pregnancy, the number of *Firmicutes* in the intestine increased, whereas the number of *Bacteroides* and *Actinomycetes* decreased [32]. The increase in the number of *Firmicutes* can promote the metabolism of carbohydrates such as fructose, galactose, mannitol, starch, and sucrose in the

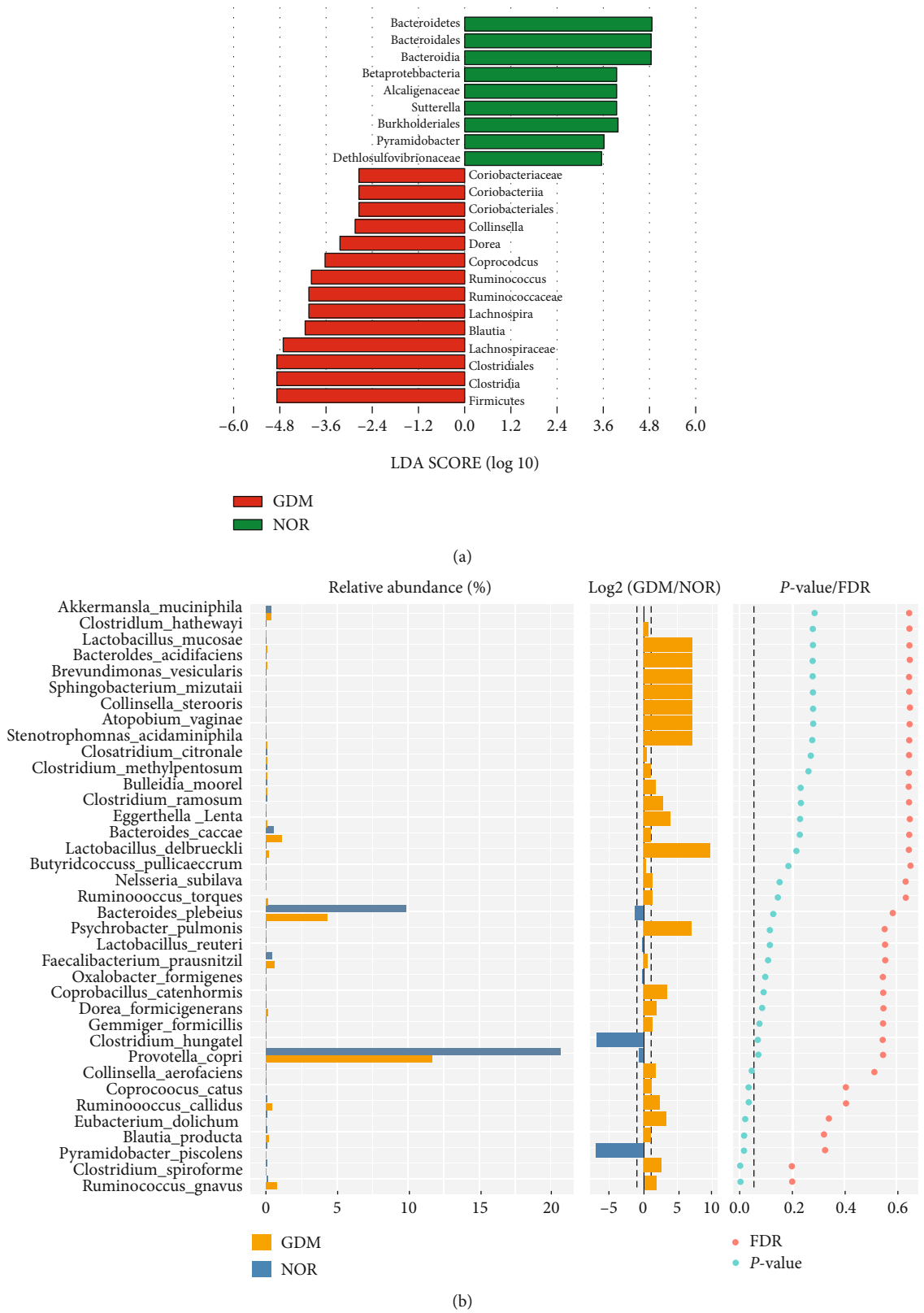


Figure 4: Analysis of the differences in intestinal flora species between the different groups. (a) LDA analysis of the NOR and GDM groups; (b) the NOR and GDM groups difference analysis of the Wilcoxon rank-sum test at the species level.

Table 2: NOR group vs. GDM group difference bacteria analysis at the species level.

Strain	NOR group (n, 29)	GDM group (n, 23)	P
Blautia_producta	0.12 ± 0.28	0.23 ± 0.30	0.01
Clostridium_spiroforme	0.002 ± 0.009	0.01 ± 0.03	0.004
Collinsella_aerofaciens	0.02 ± 0.03	0.06 ± 0.08	0.04
Coprococcus_catus	0.05 ± 0.08	0.13 ± 0.16	0.03
Eubacterium_dolichum	0.002 ± 0.003	0.02 ± 0.03	0.02
Pyramidobacter_piscolens	0.001 ± 0.003	0	0.01
Ruminococcus_callidus	0.09 ± 0.12	0.42 ± 0.57	0.03
Ruminococcus_gnavus	0.17 ± 0.13	0.66 ± 0.88	0.003

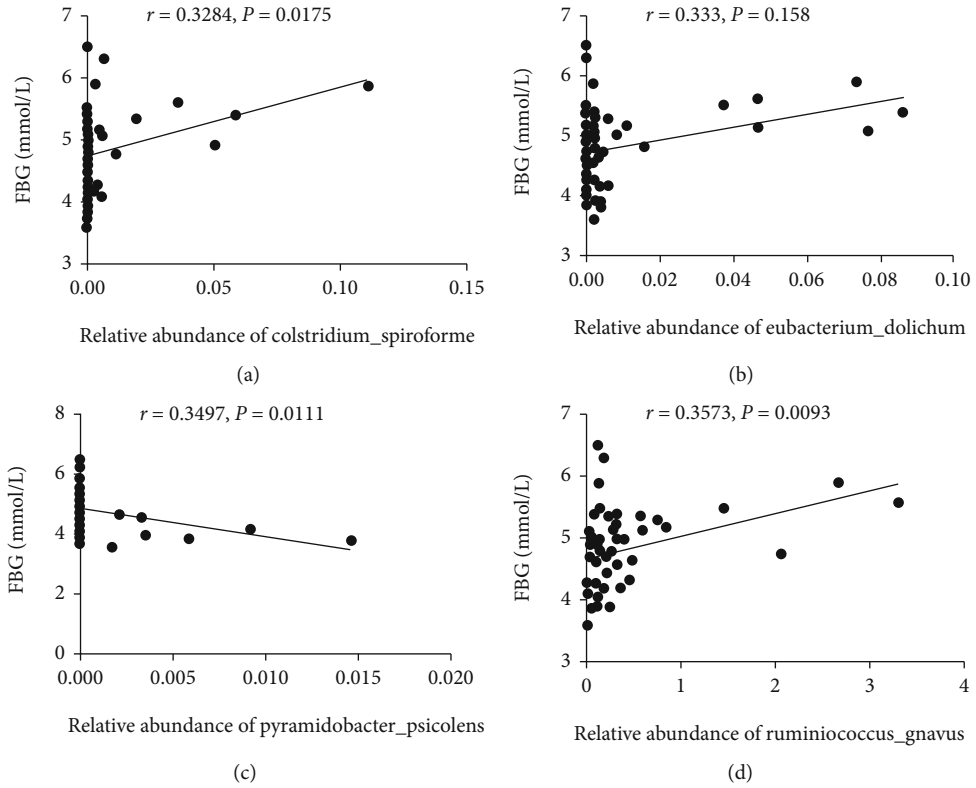
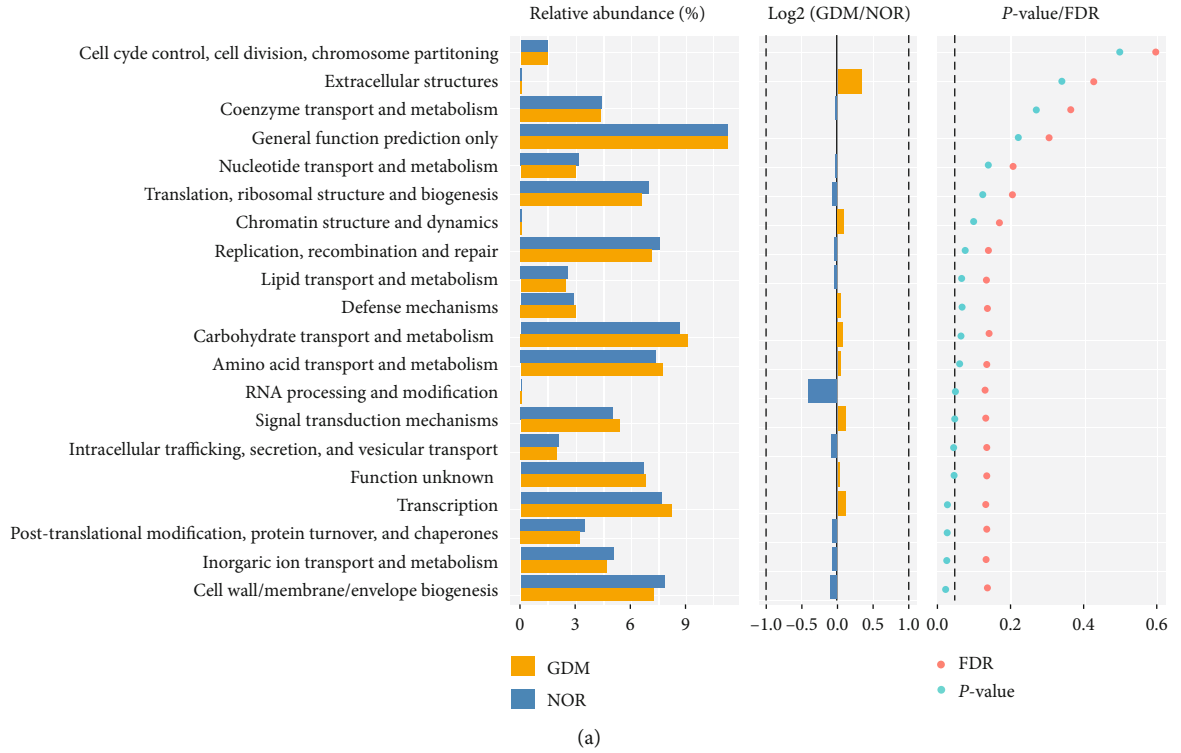


Figure 5: The correlation analysis between the relative abundance of gut bacteria with FBG.

intestines, thereby aggravating hyperglycemia, whereas the effect of *Bacteroidetes* is the opposite [33–36]. An imbalance of the intestinal flora in the second and third trimesters could affect the absorption of nitrogenous substances, block the bile acid cycle, affect the metabolism of sugar and fat in the body, and ultimately lead to GDM. Changes in the intestinal flora during pregnancy are similar to those of obese patients. In middle and late pregnancy, to meet the needs of fetal growth and development, pregnant women antagonize the increase in insulin-like substances in their bodies. The sensitivity of pregnant women to insulin decreases with increasing gestational age, and the insulin requirement increases accordingly. There is a certain degree of physiological insulin resistance (IR) in the body's glucose metabolism itself [37], and IR is also an essential mechanism of obesity and GDM.

Further analysis of the differences in species showed that there were significant differences in the abundance of eight strains in the NOR and GDM groups at the species level, whereas there were significant differences in the abundance of five strains in the G and LG groups. Additionally, the differential strains between the NOR and GDM groups are different from that between the G and LG groups, suggesting that there are differences in the long and short gestational gut microbes of GDM patients in the third trimester. Upon further analysis of the correlation between the different strains and the patient's blood sugar, the results revealed that the difference between the G and LG groups has a low correlation with the blood sugar. It could be that the amounts of the different strains are low and as such not enough to affect the blood sugar. The relative abundances of *Clostridium*



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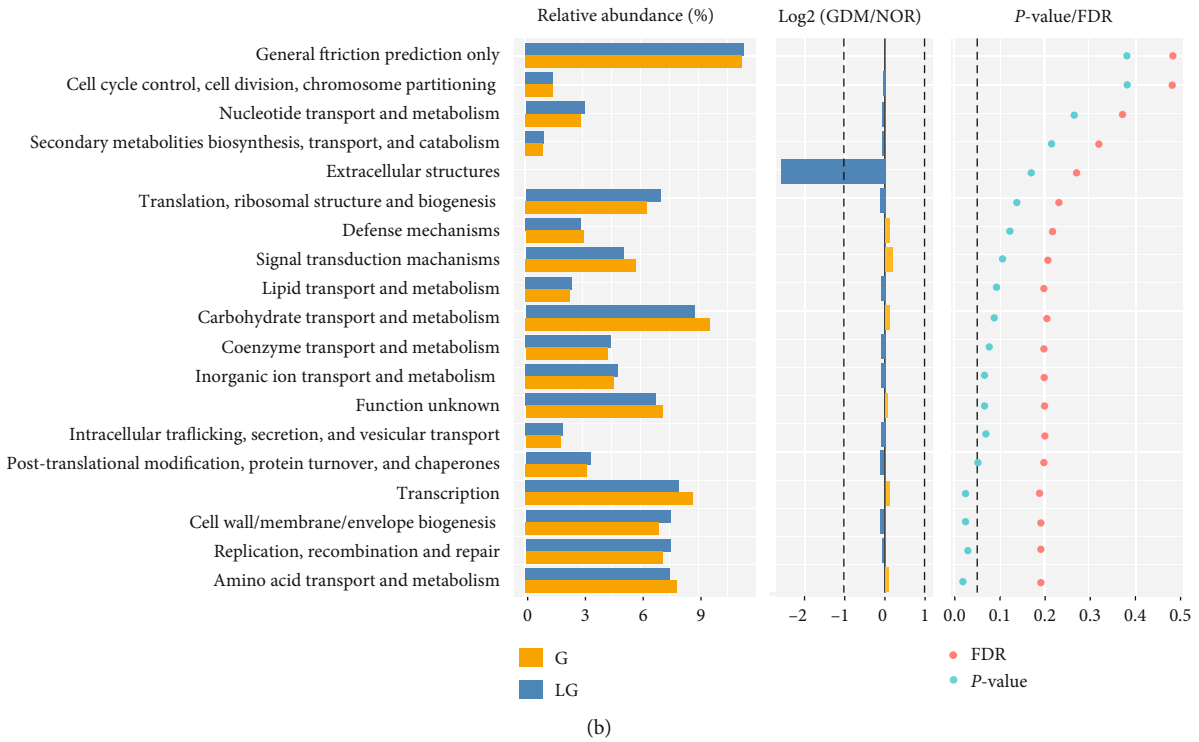
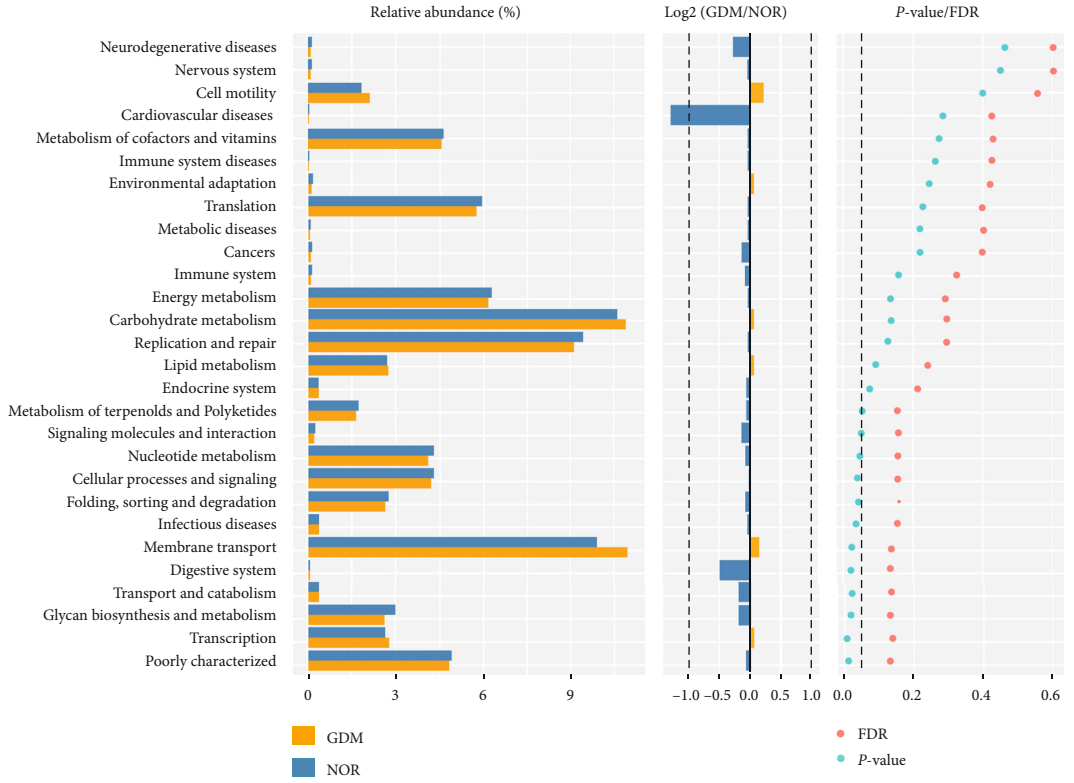
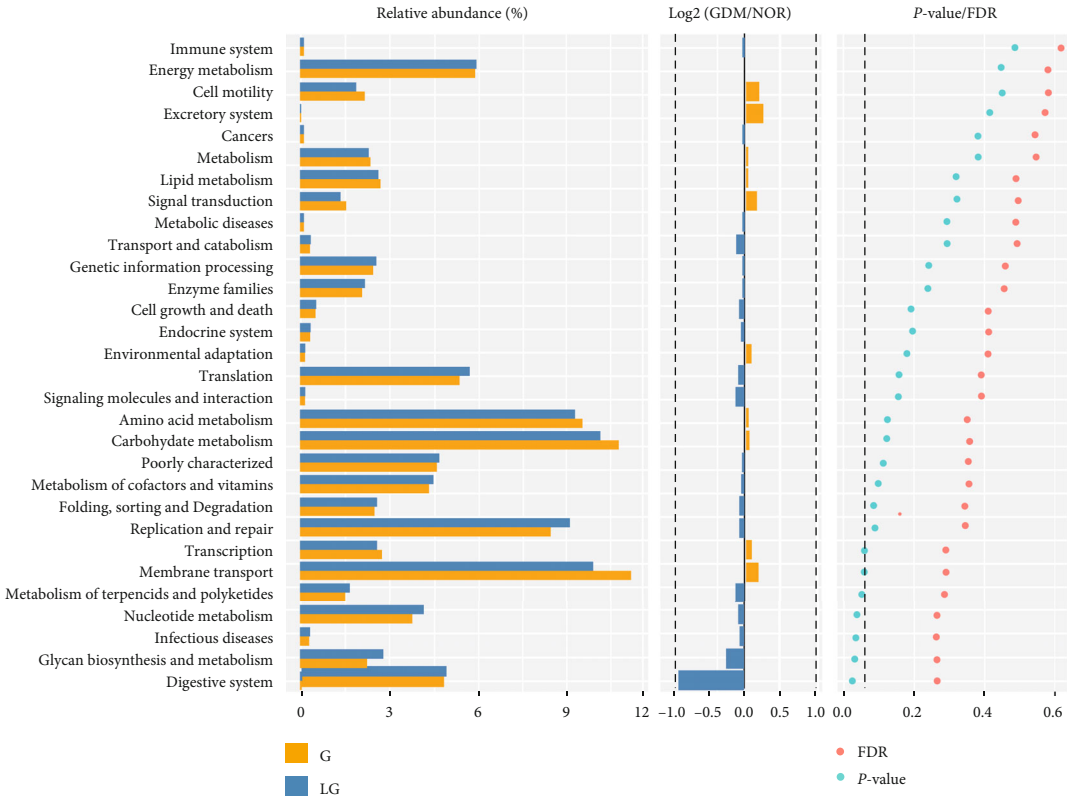


(b)

Figure 6: Continued.



(c)



(d)

Figure 6: Analysis of the difference in intestinal microbial function between different groups. (a, b) Comparative analysis of COG level 2 difference between the NOR and GDM groups and the G and LG groups; (c, d) comparative analysis of KEGG level 2 difference between the NOR and GDM groups and the G and LG groups.

spiroforme, *Eubacterium dolichum*, and *Ruminococcus gnavus* in the NOR and GDM groups were positively correlated with FBG, and *Pyramidobacter piscolens* was negatively correlated with FBG. Studies have shown that *Ruminococcus* can cause cells to absorb too much sugar, which can lead to obesity or overweight [38, 39]. However, some researchers believe that *Ruminococcus* can promote the metabolism of bile acids, which can bind with GBPAR1 and bile acid receptors (FXR) to help regulate the homeostasis of the intestinal flora and prevent intestinal microbes from releasing excessive lipopolysaccharides, which helps insulin to lower the blood sugar [40, 41]. The *Ruminococcus gnavus* species found in this paper is a species belonging to the genus *Ruminococcus*. The results of this study suggest that it is positively correlated with FBG during late pregnancy in patients with GDM.

Subsequently, changes in the specific functions caused by the changes in GDM intestinal microbes were analyzed. The different analyses showed that the intestinal microbes of the NOR and GDM groups were involved in the biosynthesis and metabolism, digestion, classification, and degradation of polysaccharides. There are differences in other biological effects, suggesting that there is a connection between the occurrence and development of GDM, and further molecular experiments are warranted to study the mechanism.

5. Conclusion

In a nutshell, with the development of 16S rDNA high-throughput sequencing, metagenomics, metabonomics, and other technologies, the research on intestinal flora and GDM has gradually deepened. Intestinal flora and metabolites have passed various pathogenic factors, inducing low-grade chronic inflammation and endotoxemia, causing IR, changing the pathway of bile acid metabolism, etc., comprehensively affecting the occurrence and development of GDM. The research on intestinal flora might adjust the dietary structure, prebiotics or probiotic preparations, and other programs for the treatment of GDM to realize the early prevention of GDM and personalized treatment and reduce the adverse pregnancy outcome for mothers and children. With further in-depth research on the relationship between gestational diabetes and the intestinal flora, it is believed that in the near future, beneficial bacteria can be supplemented to prevent and treat gestational diabetes, which is of great significance in promoting mother and child health and reducing the occurrence of diabetes.

Abbreviations

GDM: Gestational diabetes mellitus
OGTT: Oral glucose tolerance test
rRNA: Ribosomal RNA
FBG: Fasting blood glucose
OTU: Operational taxonomic unit
LEfSe: Linear discriminant analysis effect size
PLS-DA: Partial least squares discrimination analysis
TC: Total cholesterol
TG: Triacylglycerol
HDL-C: High-density lipoprotein cholesterol
LDL-C: Low-density lipoprotein cholesterol.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

G.X.L., S.H.C., P.Y., and W.L.G. researched and analyzed the data and wrote the manuscript. E.W.Y., Y.J.X., and S.H.C. designed the study and provided substantial contribution to the overall discussion. T.X.Z. and S.H.G. edited the manuscript. J.Y. is responsible for the integrity and the accuracy of the data analysis.

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Research Article

Clinical and Functional Characteristics of a Novel *KLF11* Cys354Phe Variant Involved in Maturity-Onset Diabetes of the Young

Yujing Sun,[1,2,3] **Jingru Qu**,[1,2,3] **Jing Wang**,[1,2,3] **Ruxing Zhao**,[1,2,3] **Chuan Wang**,[1,2,3] **Li Chen**,[1,2,3] **and Xinguo Hou**[1,2,3]

[1]*Department of Endocrinology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250012, China*
[2]*Institute of Endocrine and Metabolic Diseases of Shandong University, Jinan, 250012 Shandong Province, China*
[3]*Jinan Clinical Research Center for Endocrine and Metabolic Diseases, Jinan, 250012 Shandong Province, China*

Correspondence should be addressed to Li Chen; chenli3@medmail.com.cn and Xinguo Hou; houxinguo@sdu.edu.cn

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Background. Mutations in human *KLF11* may lead to the development of maturity-onset diabetes of the young 7 (MODY7). This occurs due to impaired insulin synthesis in the pancreas. To date, the clinical and functional characteristics of the novel *KLF11* mutation c.1061G > T have not yet been reported. *Methods*. Whole-exon sequencing was used to screen the proband and family members with clinical suspicion of the *KLF11* variant. Luciferase reporter assays were used to investigate whether the *KLF11* variant binds to the insulin promoter. Real-time PCR, western blotting, and glucose-stimulated insulin secretion (GSIS) analysis were used to analyze the *KLF11* variant that regulates insulin expression and insulin secretion activity in beta cell lines. The Freestyle Libre H (Abbott Diabetes Care Ltd) was used to dynamically monitor the proband daily blood glucose levels. *Results*. Mutation screening for the whole exon genes identified a heterozygous *KLF11* (c.1061G > T) variant in the proband, her mother, and her maternal grandfather. Cell-based luciferase reporter assays using wild-type and mutant transgenes revealed that the *KLF11* (c.1061G > T) variant had impaired insulin promoter regulation activity. Moreover, this variant was found to impair insulin expression and insulin secretion in pancreatic beta cells. The proband had better blood glucose control without staple food intake ($p < 0.05$). *Conclusions*. Herein, for the first time, we report a novel *KLF11* (c.1061G > T) monogenic mutation associated with MODY7. This variant has impaired insulin promoter regulation activity and impairs insulin expression and secretion in pancreatic beta cells. Therefore, administering oral antidiabetic drugs along with dietary intervention may benefit the proband.

1. Introduction

KLF11 was first cloned in a human cystic fibrosis pancreatic adenocarcinoma cell line by Urrutia et al. in 1998 [1]. *KLF11* is a pancreas-enriched transcription factor that has elicited significant attention from researchers due to its role as a negative regulator of exocrine cell growth both *in vitro* and *in vivo* [2]. Previous studies have shown that *KLF11* is involved in the pathophysiological process of diabetes development [3–5]. Moreover, mutations in human *KLF11* may lead to the development of maturity-onset diabetes of the young 7 (MODY7). Neve et al. were the first to study MODY7 [2]. In 2005, genetic screening on two probands with a family history of early onset type 2 diabetes revealed that one proband had type 2 diabetes, while the other had reduced glucose tolerance [2]. This is due to the impairment of insulin synthesis from the pancreas. Genetic analysis of *KLF11* has revealed two rare variants (Ala347Ser and Thr220Met) that are segregated in families with early onset type 2 diabetes and significantly impair its transcriptional activity [2].

In 2019, a novel *KLF11* variant (p.His418Gln) was reported; this was associated with early childhood-onset type 1B diabetes [6]. As such, *KLF11* is a valid candidate gene to

determine the genetic predisposition to early onset and type 2 diabetes, as defects in this gene may lead to early onset or polygenic type 2 diabetes [7]. In addition to *KLF11*, there may be other unknown factors that need further exploration. However, *KLF11*, due to its role as a MODY gene, is a potential therapeutic target for adult-onset diabetes.

In this study, we report a novel *KLF11* variant associated with MODY7 and explore its clinical features, possible pathogenesis, optimal treatment, and functional characteristics. Furthermore, we also investigated whether dietary intervention may benefit diabetic patients with *KLF11* mutation.

2. Materials and Methods

2.1. Patients

2.1.1. Proband. The proband (III-4, Figure 1) was a female subject aged 23 years, with normal development and moderate nutritional status. However, one physical examination on September 2nd, 2018, revealed that the proband's fasting blood glucose level was 13.9 mmol/L. Immediately prior to admission to the local hospital, the proband's fasting blood glucose level was 10.36 mmol/L, and HbA1c levels were 11.5%. Due to the high blood glucose level, the doctor treated her with insulin and oral hypoglycemic agents. One month later, the proband's blood glucose control worsened after gradually adjusting the treatment to saxagliptin (2.5 mg/day) and voglibose (0.6 mg/day), which was followed by admission to the QiLu Hospital for further treatment. The patient reported no polydipsia, polyuria, weight loss, or blurred vision and no numbness of the limbs, fatigue, or discomfort during this time.

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the ethics committee of the QiLu Hospital of Shandong University (No: KYLL-2020(KS)-069). All the subjects included in our study voluntarily signed an informed consent form, which was reviewed by the ethical committee.

2.1.2. Family History. There was no history of diabetes on the paternal side of the family, whereas there was one diabetic patient among maternal relatives (maternal grandfather).

2.1.3. Physical Examination. The proband's height was 168.5 cm; she weighed 54 kg and had a body mass index (BMI) of 19.02 kg/m^2. Moreover, the proband did not exhibit any obvious abnormalities of the heart, lungs, or abdomen, and there was no edema in either of the lower limbs.

2.1.4. Treatment Methods. Due to the unsatisfactory control of both fasting and postprandial blood glucose levels, the treatment was changed to insulin glargine injections (10 units at bedtime), oral saxagliptin (2.5 mg; once/day), and oral voglibose (0.2 mg; thrice/day).

2.1.5. Clinical Data Collection. The subject's family provided informed consent and was enrolled in September 2018. We collected comprehensive clinical data, such as results of physical examinations, medical history, pedigree, and levels of related metabolism products.

2.2. Mutation Analysis. Peripheral blood was collected from four family members (the proband, her parents, and her maternal grandfather). The EDTA anticoagulation and E.Z.N.A.® Blood DNA Mini Kit (omega Bio-Tek, Inc. D3392) were used to extract the genomic DNA from peripheral blood leukocytes, which was then sent to Beijing Fujun Gene Biotechnology Co., Ltd. for whole-exome sequencing.

2.3. In Silico Analysis of the KLF11 Variant. Using the phyre2 server, the three-dimensional (3D) structures of wild-type KLF11 (WT-KLF11) and its variant (Cys354Phe-KLF11) were predicted using the threading method and comprehensively analyzed through head-to-head comparison of the final models. The WT-KLF11 and Cys354Phe-KLF11 3D structural models were established as reference models [8]. Structure visualization was performed using the SAVES v5.0 server.

2.4. Plasmid Information. The plasmid vector encoding the insulin promoter sequence pGL3-basic-INS, which was first reported by Bernadette Neve et al. [2], was synthesized by Biosune Biotechnology (Shanghai) Co., Ltd. The pCDNA3.1-WT-KLF11 plasmid was purchased from Biosune Biotechnology (Shanghai) Co., Ltd. The pCDNA3.1-C354F-KLF11 plasmid was constructed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA), according to the manufacturer's instructions. All constructs were verified by sequencing on an ABI 3730xl sequencer. The construct was completely sequenced and used as the template in other cloning designs.

2.5. Western Blotting. Human embryonic kidney (HEK) 293 cells were cotransfected with plasmids encoding Cys354Phe-KLF11 and WT-KLF11 and were cultured as previously described [9]. At 48 h posttransfection, the cells were harvested and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed using a mouse monoclonal anti-KLF11 primary antibody (Origene, Cat: TA811001s, 1 : 1000) and horseradish peroxidase-conjugated goat anti-mouse IgG polyclonal secondary antibody (Zhongshan Golden Bridge, Cat: ZB-2305, 1 : 10000). Protein bands were detected using a chemiluminescence kit (Millipore, CA, USA, WBKLS0050) and imaged using a chemiluminescence imaging system (Shanghai Qinxiang Scientific Instrument Co., Ltd.).

2.6. Luciferase Reporter Assay. HEK 293 cells were seeded in 96-well culture plates (10,000 cells/well in 200 μL of culture media) and cultured for 24 h. Then, each cell group was transfected separately with a pRL-TK plasmid, pGL3-basic-INS, and pCDNA3.1-WT-KLF11 or pCDNA3.1-C354F-KLF11 using LipofectamineTM 2000(Invitrogen, cat:11668-027), according to the manufacturer's instructions. At 48 h posttransfection, the dual-luciferase reporter assay was

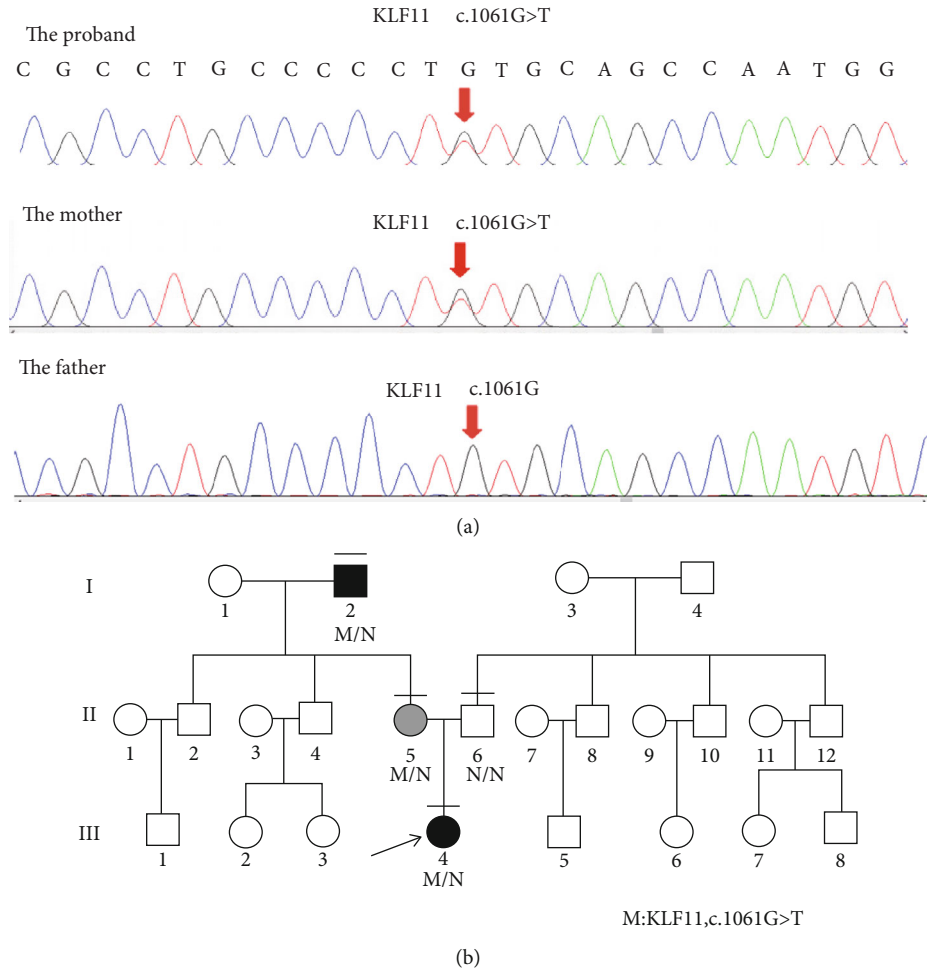

Figure 1: Partial sequence diagram of *KLF11* and the pedigree of the studied family members. (a) Partial sequence diagram of *KLF11*. A heterozygous c.1061G > T transition mutation, causing the substitution of cysteine by phenylalanine at codon 354 is shown using an arrow (GenBank accession number: NM_003597.4). (b) The pedigree of the study family. Women are represented using circles and men, using squares. The black symbols indicate individuals with diabetes. The grey symbol indicates individuals with prediabetes. The proband is denoted by an arrow. The horizontal lines indicate individuals who underwent molecular analysis. The p.Cys354Phe variant of *KLF11* was identified in I-2, II-5, and III-4. The N symbols denote the people that carry the WT gene, and the M symbols denote the people that carry the Cys354Phe variant.

performed using the Luciferase Reporter Assay Kit (Promega, E1910).

2.7. Insulin Secretion and Insulin Content Assay. INS1 cells were cotransfected with plasmids encoding Cys354Phe-KLF11 and WT-KLF11 to investigate insulin secretion and insulin contents, as described previously [10]. Insulin secretion was determined using a static incubation method under conditions of 5% CO_2 and 95% air at 37°C, as previously described [11, 12]. Cells were seeded at a density of 2×10^5 cells in 24-well plates and cultured in 1 mL of Dulbecco's Modified Eagle Medium (DMEM, 25 mmol/L glucose). After 48 h, the medium was removed, and cells were washed once with HEPES-balanced Krebs Ringer Bicarbonate Buffer (119 mmol/L NaCl, 4.74 mmol/L KCl, 2.54 mmol/L $CaC1_2$, 7.4 mmol/L $MgC1_2$, 1.19 mmol/L $KHPO_4$, 25 mmol/L $NaHCO_3$, 10 mmol/L HEPES, pH 7.4) containing 0.5% bovine serum albumin (BSA) without glucose. Next, the cells were preincubated in HEPES-balanced Krebs-Ringer Bicarbonate Buffer with 0.5% BSA and 5 mmol/L glucose for 30 min. After washing twice with HEPES-balanced Krebs-Ringer Bicarbonate Buffer, INS1 cells were incubated in HEPES-balanced Krebs-Ringer Bicarbonate Buffer supplemented with 0.5% BSA and varying concentrations of glucose. The media were then collected and assayed for immunoreactive insulin via an enzyme-linked immunosorbent assay (ELISA), with mouse insulin being used as a standard. A volume of 200 μL of 1 mol/L NaOH was added to each well to solubilize the cells in order to determine the cellular protein contents using an ELISA assay kit (Millipore, EZRMI-13 K). For the measurement of cellular insulin content, 1 mL of acid ethanol was added to the wells, which were then sealed with a pressure-sensitive film. The extract was collected after 24 h incubation at 4°C and was then diluted and assayed by ELISA.

2.8. Real-Time PCR. INS1 cells were isolated using the TRIzol reagent (Takara, T9108). The cDNA was generated using the

HiScript Q RT SuperMix for qPCR (Takara, DRR047S), and the real-time PCR assays were conducted with an LC480 Light Cycler using the following primer sequences: Ins1 forward primer, GAAGAGGCCATCAAGCAGATCACT; Ins1 reverse primer, ATTGTTCCACAATGCCACGCT; GAPDH forward primer, GCCTTCCGTGTTCCTACC; and GAPDH reverse primer, GCCTGCTTCACCACCTTC. Relative gene expression was determined using a comparative method ($2^{-\triangle\triangle CT}$). GAPDH was used as an internal standard.

2.9. Statistical Analysis. All data are presented as the means ± standard deviations (SDs) or means ± standard errors of the means (SEMs). Statistical comparisons were performed by using two-tailed, paired Student's *t*-test for datasets involving only two groups, or by using one-way ANOVA in the case of data involving more than two groups. Then, the Dunnett's and Bonferroni's post hoc tests were performed for multiple comparisons. All tests were performed using GraphPad Prism 8. Every experiment was repeated at least thrice independently. Representative experimental results are shown in the figures. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Clinical Manifestation. As the proband was first diagnosed with diabetes at the age of 23 and treated with insulin and oral hypoglycemic agents, it was crucial to identify which type of diabetes the proband had. After three weeks of treatment, the HbA1c levels were 6.6%. Therefore, this treatment plan was maintained for one and a half years, and the patient's blood glucose level was controlled and stable during this time.

3.2. Laboratory Data. Islet autoantibody screening revealed an absence of glutamic acid decarboxylase (GAD), tyrosine phosphatase antibodies (IA-1ABs), anti-insulin cell antibodies (ICA-IgG), insulin autoantibodies (IAAs), and β-cell-specific zinc transporter 8. When the proband's blood glucose level was normal and stable, an oral glucose tolerance test (OGTT) was performed simultaneously with insulin and C-peptide release experiments to assess islet function (Table 1). As the maternal grandfather of the proband was a diabetes patient, we performed genetic testing for the proband. As such, we identified a heterozygous variant of *KLF11* (c.1061G > T, p.Cys354Phe) via whole-exome sequencing. Moreover, the proband exhibited no abnormalities in the thyroid function, hepatic and renal function, blood lipid profile, or urine microalbumin.

3.3. Clinical Characteristics of Family Members and Their Genetic Testing Results. The maternal grandfather of the proband (I-2) was diagnosed with type 2 diabetes at 66 years of age and treated with oral hypoglycemic agents. His blood HbA1c level was 6.0% during his last examination (Table 2). The parents of the proband were never diagnosed with diabetes, but the mother (II-5) was found to have a fasting blood glucose level of 5.8 mmol/L at the physical examination. Genetic testing for MODY facilitates a correct diagnosis, thereby enabling treatment optimization and allowing the monitoring of asymptomatic family members. Therefore, genetic tests were conducted for the parents and maternal grandfather of the proband. As expected, the heterozygous variant of *KLF11* (c.1061G > T, p.Cys354Phe) was also identified in the mother and maternal grandfather (Figure 1(a)). In order to confirm whether the mother was an asymptomatic member, we performed an OGTT simultaneously with insulin and C-peptide release experiments (Table 1). Our results showed that the mother of the proband can be diagnosed with impaired glucose tolerance, which means she is prediabetic and may develop diabetes in the future.

Table 1: Blood glucose, insulin, and C-peptide levels of the proband and her mother.

Time (h)	Blood glucose (mmol/L)		Insulin (μIU/mL)		C-peptide (ng/mL)	
	II-5	III-4	II-5	III-4	II-5	III-4
0	5.27	6.98	3.12	8.99	0.66	0.31
0.5 h	10.47	9.02	24.1	11.27	3.97	0.54
1 h	10.91	10.3	33.38	15.1	5.63	0.95
2 h	6.57	12.43	59.63	17.45	6.37	1.44
3 h	3.38	9.81	29.38	15.2	3.07	1.49

As there are differences in the clinical phenotypes caused by the same mutation even within the same family, these results may indicate that *KLF11* mutations are associated with incomplete penetrance. In summary, our results indicate that the *KLF11* (c.1061G > T) variant is associated with diabetes in this family.

3.4. Sequencing Results and Biochemical Characterization of the KLF11 (c.1061G > T) Variant. The conserved domain of human *KLF11* consists of an extracellular region that comprises three transcriptional repressor domains (TRD) and a zinc finger domain. *KLF11*, as a member of the Sp1/KLF family, has been predicted to bind to either GC-rich or CACC sequences. The Cys354Phe-KLF11 variant was mapped to a novel hydrophobic glycine-glutamine-proline-rich domain that was observed in the corresponding region of its fly ortholog, cabut (Figure 2(a)). The KLF11 Cys354Phe variant was also mapped to the previously characterized transcriptional regulatory domain 3 (TRD3). Multiple amino acid sequence alignments using Clustal W showed that Cys354Phe-KLF11 was conserved across various species (Figure 2(b)). It was predicted that mutations in this buried site led to its exposure on the surface of the protein, thereby modifying the protein activity. In addition, due to the exposure of this site at the surface of the protein after mutation, it might be located in a larger transcriptional blocking domain and affect some transcriptional functions (Figure 2(c)).

3.5. Functional Characterization of the KLF11 (c.1061G > T) Variant. The link between the *KLF11* (c.1061G > T) variant and the putative diabetes pathophysiological process was assessed. The *KLF11* (c.1061G > T) gene variant did not affect protein expression levels (Figures 3(a) and 3(b)). The results

Table 2: Clinical characteristics of the study family.

	Proband	Maternal grandfather	Mother‡	Father‡
Birth weight (kg)	3.15	No data	No data	No data
At diagnosis				
Age (yr)	23	66	—	—
Height (cm)	168.5	172	—	—
Body weight (kg)	54	65	—	—
BMI (kg/m^2)	19.02	21.97	—	—
DKA†	No	No	—	—
Blood glucose (mmol/L)	13.9	No data	—	—
HbA1c (NGSP, %)	11.5	No data	—	—
At the latest examination				
Age (yr)	24	70	50	50
Height (cm)	168.5	172	163	173
Body weight (kg)	50	55	50	65
BMI (kg/m^2)	17.61	18.59	18.82	21.72
HbA1c (NGSP, %)	5.9	6.0	5.1	4.6
Fasting serum C-peptide (ng/mL)	0.31	0.72	0.50	0.73
Fasting serum insulin (μIU/mL)	8.99	4.74	4.09	2.65
Treatment plan	Insulin glargine injection (10 units at bedtime), saxagliptin (2.5 mg once/day), voglibose (0.2 mg three times/day)	Metformin hydrochloride 1000 mg/day; gliclazide 160 mg/day	—	—

†DKA: diabetic ketoacidosis. ‡This individual had no diabetes.

of the luciferase assays demonstrated that *KLF11* plays a role in and the transcriptional regulation of the insulin gene. The insulin promoter activity induced by the *KLF11* (c.1061G > T) variant was observed to be lower than that induced by WT-KLF11 (Figure 3(c)). Moreover, the insulin gene activation was observed to be affected in the mutant *KLF11*. To further explore the role of the *KLF11* Cys354Phe variant on β-cell function, we overexpressed the WT and variant *KLF11* in INS1 cells (using the pcDNA3.1 plasmid as the negative control and the *KLF11* (WT and Cys354Phe) plasmids) and found that the *KLF11* Cys354Phe variant decreased insulin transcription and reduced insulin secretion even after stimulation with high glucose (Figures 3(d) and 3(e)).

3.6. Exploration of KLF11 (c.1061G > T) Gene Variant Treatment. The blood glucose level of the proband was under control after treatment with insulin and oral hypoglycemic agents for one and a half years (Table 2). Due to the inconvenience of using insulin at school, the medication was adjusted to oral gliclazide sustained-release tablets (30 mg/day) combined with diet control in June 2020. We found that together with the control of staple food intake, this treatment resulted in better blood sugar control in a two-week staple diet trial. There was no significant difference in energy intake between the two weeks ($p < 0.05$, Table 3). However, there were significant differences in average daily blood glucose, fasting blood glucose, and 2 h postmeal blood glucose between the two weeks ($p < 0.05$, Table 3). There was no statistical difference in the mean amplitude of glycemic excursions (MAGE) between the two weeks, which represented blood glucose fluctuations. According to the dynamic blood glucose parameters, dietary intervention may be beneficial for the proband and help control daily glucose levels (Figures 4(a) and 4(b)).

4. Discussion

The diagnosis of MODY is challenging due to its relatively low prevalence and the overlap in presentation and clinical features between patients with MODY and those with other diabetes subtypes [13]. MODY is characterized by autosomal-dominant inheritance with a multigenerational family history of diabetes, onset before 25 years of age, and the absence of pancreatic autoimmunity and insulin resistance [14]. In this study, we identified a *KLF11* variant in three individuals belonging to one family via whole-exome sequencing. Among them, two individuals (including one young adult) developed diabetes. The proband exhibited hyperglycemia at 23 years of age and was observed to be negative for islet cell autoantibodies. The maternal grandfather developed type 2 diabetes at the age of 66. The mother was considered non-diabetic,but had delayed insulin secretion

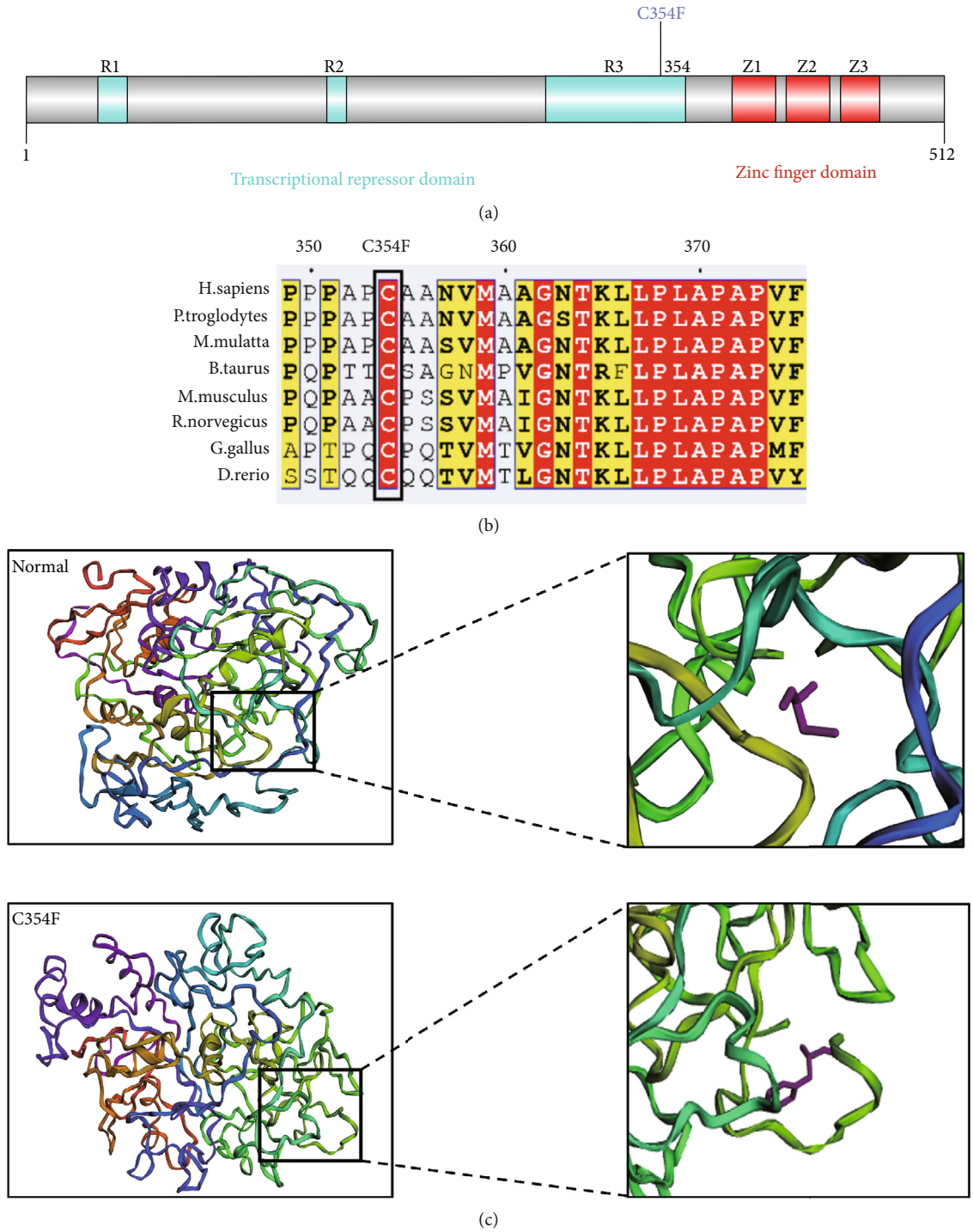

Figure 2: Sequencing analysis and 3D structure of the mutant protein. (a) KLF11 structure domains. Mutations at the protein level are indicated below the 3RDB domain. (b) Cross-species conservation of Cys354Phe-KLF11. (c) Protein structure prediction of KLF11 (WT and Cys354Phe).

via OGTT and islet function test, which is one of the characteristics of diabetes. The father was considered healthy. In fact, strict adherence to these guidelines confers high specificity but low sensitivity in identifying MODY subjects, as more than half of patients with confirmed mutations identified in European countries do not meet these clinical criteria for referral. As such, adherence to current guidelines will continue to lead to the misdiagnosis of a large proportion of patients with MODY [15, 16]. Furthermore, by extending MODY diagnostic testing beyond current guidelines, Owen et al. [17] identified MODY subjects with clinical features that are not characteristic of MODY, including the absence

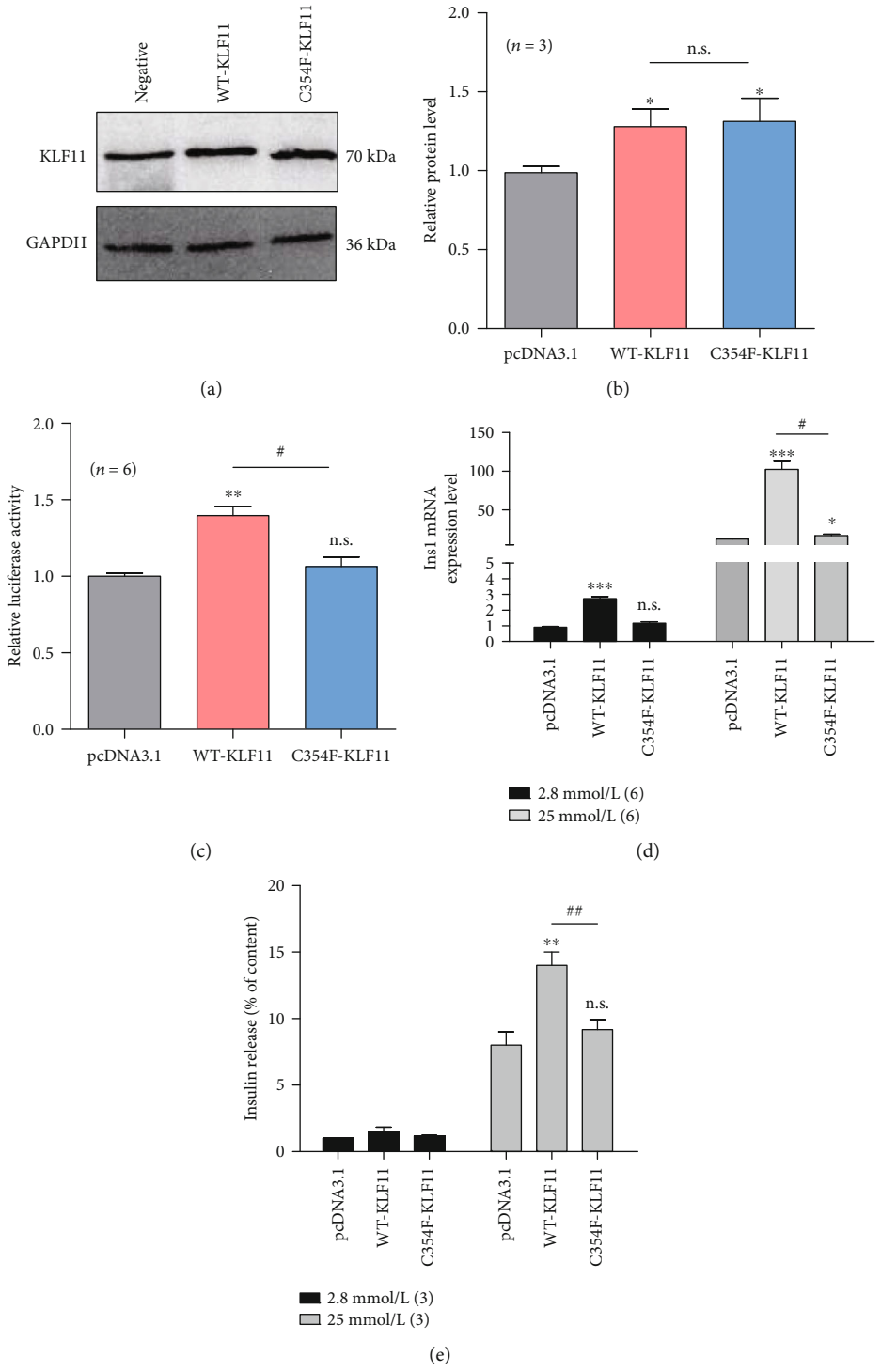

Figure 3: Functional analysis of the KLF11 Cys354Phe variant. (a) Protein expression of KLF11 (WT and Cys354Phe). Cell lysates of KLF11-expressing cells were used. Protein production was tested via western blotting. (b) Histogram of the KLF11 protein expression level analysis. (c) Luciferase assays of HEK 293 cells transfected with each KLF11 expression vector (WT and Cys354Phe). ** denotes $p < 0.01$ ($p = 0.0031$) for the KLF11 WT plasmid compared to the empty plasmid; n.s. denotes $p > 0.05$ ($p = 0.3899$) for KFL11/C354F compared to the empty plasmid; # denotes $p < 0.05$ ($p = 0.0176$) for KFL11/C354F compared to KLF11 WT. (d–f) INS1 cells were transfected with the KLF11-WT or KLF11-Cys354Phe plasmids for 24 (d) or 48 h (e). (d) qRT-PCR was conducted to determine the Ins1 mRNA levels after stimulation with 2.8 mmol/L glucose (low) or 20 mmol/L glucose (high) for 24 h. (e) Insulin secretion levels in INS1 cells were analyzed via glucose-stimulated insulin secretion assay using ELISA after stimulation with 2.8 mmol/L glucose (low) or 20 mmol/L glucose (high) for 2 h. Note: *n* denotes the number of experiments. ** denotes $p < 0.01$ ($p = 0.0018$) for KLF11 WT compared to the empty plasmid; n.s. denotes $p > 0.05$ ($p = 0.1836$) for KFL11/C354F compared to the empty plasmid; ## denotes $p < 0.01$ ($p = 0.0027$) for KFL11/C354F compared to KLF11 WT.

Table 3: The mean and standard deviation of blood glucose and daily energy intake of the proband between the two weeks.

Variable		First week with staple foods		Second week without staple foods		p value
		Mean	SD	Mean	SD	
Blood glucose (mmol/L)	Daily average	5.65	0.49	4.16	0.54	0.000
	Fasting	5.21	0.52	4.09	0.78	0.01
	2 h after breakfast	7.03	0.38	5.15	1.14	0.004
	2 h after lunch	5.8	1.13	4.2	1.20	0.054
	2 h after dinner	7.12	1.91	4.87	0.86	0.021
Mean amplitude of glycemic excursions (MAGE)		2.88	0.83	2.93	0.87	0.918
Daily energy intake (kilocalories)		918.28	287.90	1023.00	507.61	0.643

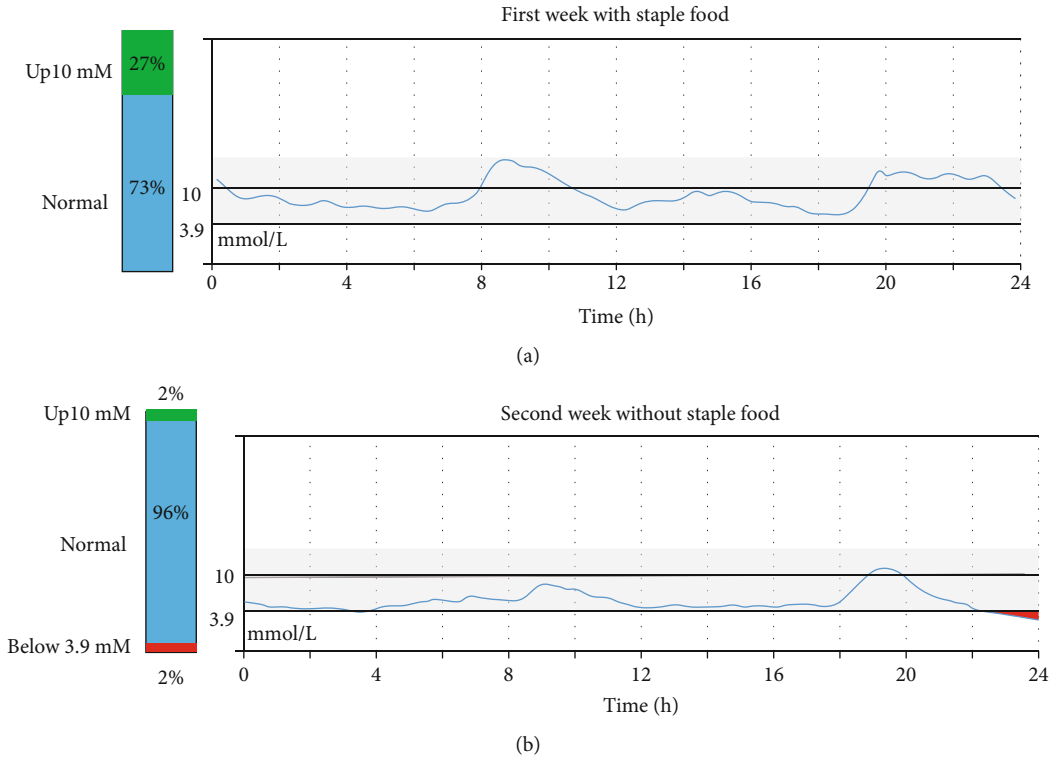

Figure 4: Representative data of the scanning dynamic glucose monitoring report for the proband with and without staple food intake. The proband wore the Freestyle Libre H (Abbott Diabetes Care Ltd.) for the dynamic monitoring of the blood glucose levels for 14 days. The proband needed to maintain normal staple food intake during the first week, while no staple food intake was allowed during the second week; the amount of calories for each meal was recorded through the "mint app" (China) on the phone. No exercise adjustments were performed in these two weeks. After 14 days, the data were analyzed to obtain the following results: (a) representative data of the scanning dynamic glucose monitoring report for the proband with staple food intake. The proband was shown to have a glucose level above 10 mmol/L for approximately 27% of the day; (b) representative data of the scanning dynamic glucose monitoring report for the proband without staple food intake. The proband was shown to have glucose levels above 10 mmol/L for only about 2% of the day.

of a family history of diabetes. Therefore, the diagnosis of MODY for the proband is definite, even without a typical family history.

It is well known that diabetes is the result of a combination of genetic and environmental risk factors. Among them, epigenetics plays a vital role in mediating the interaction between environmental and genetic factors. Epigenetics also has an intergenerational effect, that is, some extragene information from the grandparents will be passed to the grandchildren, and marks will be burned on the grandchildren, who show the corresponding characteristics. These factors may contribute to the earlier onset of diabetes.

Genetic studies have found that missense mutations in the *KLF11* gene lead to the development of late-onset diabetes [7]. This is due to the fact that the mutations affect *KLF11*-binding promoters and activation of the bladder protein gene. Moreover, after mutations in the cis-acting element of *KLF11* were found to inhibit *KLF11*-induced activation of the insulin gene, leading to a decrease in the biosynthesis of insulin in the body. The KLF11 protein is a zinc

finger transcription factor that binds to SP1-like sequences in the promoter region of the ε- and γ-globin genes [18]. Three transcript variants encoding two different isoforms have been found for *KLF11* mRNA, and their proteins are expressed in pancreatic β-cells [10]. This binding increases its repression and impairs the activation of insulin promoters [19]. In addition, defects in this gene cause MODY7. Furthermore, Neve et al. identified two rare *KLF11* variants (Ala347Ser and Thr220Met) in families with early onset type 2 diabetes, which were shown to significantly impair the transcriptional activity of *KLF11*. Furthermore, they discovered a frequent polymorphic Gln62Arg variant that was significantly associated with type 2 diabetes mellitus in North European populations [2]. Ushijima et al. identified a heterozygous *KLF11* (p.His418Gln) variant in a family that was clinically diagnosed with early childhood-onset type 1B diabetes [6]. These two diabetes-associated studies revealed the effects of a loss of *KLF11* protein function.

Multiple amino acid sequence alignments showed that KLF11 Cys354Phe (C354F) was conserved across various species. Moreover, the cells transfected with the *KLF11*-WT and mutant plasmid constructs exhibited no statistically significant differences (data not shown). Moreover, there were no significant differences in the protein expression levels between the *KLF11*-WT- and *KLF11*-C354F-transfected cells. Notably, the *KLF11* (c.1061G > T) variant induced lower insulin promoter activity than *KLF11*-WT. This indicates that the mutant affects the *KLF11*-induced activation of the insulin gene. It is predicted that mutations at this buried site may lead to its exposure on the surface of the protein, thereby altering the protein activity [20, 21]. In addition, due to the exposure of this site at the surface of the protein after mutation, it might be possible that the site is located in a larger transcriptional blocking domain, thereby affecting the transcriptional functions of insulin [22]. Further cell function studies showed that the *KLF11* Cys354Phe variant decreased insulin transcription and reduced insulin secretion even upon stimulation with high glucose. Moreover, dysregulation of *KLF11* also affected the insulin content of cells.

Children and adolescents diagnosed with diabetes may initially be treated with insulin, and this regimen often continues even after the stabilization of glycemia. However, in some patients with MODY, hyperglycemia can be controlled by prescribing oral antidiabetic drugs (e.g., sulfonylureas), without the use of insulin [23]. In fact, standard treatments have not been established for most diabetes subtypes due to the low number of cases and lack of data confirming treatment efficacy. Administration of insulin therapy from the initial phase of MODY7 has been described in previous study [6], which is consistent with the treatment received by the proband in our study. In this case, using insulin (using and storage defects) also led to inconvenience for the proband. Moreover, after treatment with sulfonylureas and when the total energy intake remained stable by reducing the intake of staple foods, we found that the proband's blood glucose levels were better controlled. Therefore, oral antidiabetic drugs and dietary intervention may benefit diabetic patients with *KLF11* mutations and help them control their daily glucose levels. The following regimen and a healthy lifestyle are very important factors in the disease control for patients with type 2 diabetes.

5. Conclusions

In summary, in this study, we successfully identified the *KLF11* (c.1061G > T) variant via whole-exome sequencing, which was shown to cause MODY7 in a 23-year-old female. This study is the first to demonstrate that *KLF11* (c.1061G > T) variants are involved in the pathogenesis of MODY7. Epigenetic factors may contribute to the earlier onset of diabetes. Moreover, we showed that the administration of oral antidiabetic drugs and dietary interventions were beneficial for the proband and helped control the daily glucose levels. Our study also has a few limitations. Further studies, such as animal experiments, are needed to explain the association between altered *KLF11* function and the diabetes pathogenesis and severity. In addition, new dietary intervention and treatment methods need to be developed for MODY.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

XGH and LC are involved with the study design. CW, RXZ, and JW are involved in the study conduct. Original draft preparation is collected by SYJ, QJR, and JW. SYJ and QJR drafted and revised the manuscript. Yujing Sun and Jingru Qu contributed equally to this work.

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Research Article

Family History of Diabetes and the Effectiveness of Lifestyle Intervention on Insulin Secretion and Insulin Resistance in Chinese Individuals with Metabolic Syndrome

Haiqing Zhu,[1] **Xiaoping Chen**,[2] **Bo Zhang**,[2] **Wenying Yang**,[2] **and Xiaoyan Xing**[2]

[1]*Department of Endocrinology, Emergency General Hospital, Beijing, China*
[2]*Department of Endocrinology, China-Japan Friendship Hospital, Beijing, China*

Correspondence should be addressed to Xiaoyan Xing; xingxy221@126.com

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Aims. The current study aims to explore if a family history of diabetes can influence the efficiency of lifestyle intervention on insulin secretion and study the insulin resistance in Chinese men and women with metabolic syndrome in a cohort with a 2-year follow-up. *Methods*. 151 individuals (90 individuals did not have a family history of diabetes (DMFH (-)) and 61 with a family history of diabetes (DMFH (+)) with metabolic syndrome participated in the lifestyle intervention program at baseline and finished with 1-year follow-up. 124 individuals have two-year follow-up data. A family history of diabetes was ascertained by self-report. Lifestyle interventions were individual sessions on lifestyle changes. *Results*. During the 1-year follow-up, Ln Insulinogenic index ($\Delta_{\text{baseline}-1\text{year}} = 0.29 \pm 0.65$, $P = 0.001$) and 30-min glucose ($\Delta_{\text{baseline}-1\text{year}} = -0.41 \pm 1.71$, $P = 0.024$) changed significantly in the DMFH(-) group; in the DMFH(+) group, Ln ISIm ($\Delta_{\text{baseline}-1\text{year}} = -0.22 \pm 0.60$, $P = 0.022$) and 30-min glucose ($\Delta_{\text{baseline}-1\text{year}} = 0.53 \pm 1.89$, $P = 0.032$) changed significantly, and there was no significant change of other parameters. The change of 30 min glucose during a 1-year intervention has shown a significant difference between the two groups ($P = 0.002$). During the 2 years intervention, Ln Insulinogenic index changed significantly in the DMFH(-) group ($\Delta_{\text{baseline}-1\text{year}} = 0.33 \pm 0.66$, $P < 0.001$ and $\Delta_{\text{baseline}-2\text{year}} = 0.43 \pm 1.17$, $P = 0.034$). Fasting insulin ($\Delta_{\text{baseline}-2\text{year}} = 2.95 \pm 8.69$, $P = 0.034$), 2 h insulin ($\Delta_{\text{baseline}-2\text{year}} = 23.75 \pm 44.89$, $P = 0.002$), Ln HOMA-B ($\Delta_{\text{baseline}-2\text{year}} = 0.43 \pm 1.02$, $P = 0.009$), Ln HOMA-IR ($\Delta_{\text{baseline}-2\text{year}} = 0.53 \pm 1.04$, $P = 0.002$), Ln ISIm ($\Delta_{\text{baseline}-2\text{year}} = 0.52 \pm 0.95$, $P = 0.004$), and Ln Insulinogenic index ($\Delta_{\text{baseline}-2\text{year}} = 0.66 \pm 1.18$, $P = 0.047$) changed significantly after 2 years of intervention, compared to the baseline in the DMFH(+) group. The change of Ln ISIm ($P = 0.023$), fasting ($P = 0.030$), and 2 h insulin ($P = 0.007$) during the 2-year intervention has shown a significant difference between the two groups. Family history of diabetes was related with a 0.500 unit increase in 2-year ISIm ($P = 0.020$) modified by lifestyle intervention adjusted for age, baseline BMI, sex, and baseline waist circumference and a 0.476 unit increase in 2-year ISIm ($P = 0.027$) with extra adjustment for weight change. *Conclusions*. Patients with a family history of diabetes benefit more from lifestyle intervention in regard to insulin resistance than those without a family history of diabetes adjusting for age, baseline BMI, sex, baseline waist circumference, and weight change.

1. Introduction

Metabolic syndrome (MetS) is a collection of obesity, hypertension, dyslipidemia, and glucose intolerance and significantly increases the risk of type 2 diabetes mellitus (T2DM). MetS is characterized by insulin resistance and hyperinsulinemia, which lead to the deterioration of β cell function [1].

Lifestyle interventions are promising strategies to stop or delay the occurrence of T2DM [2]. Both genetic factors and adherence to lifestyle management influence the efficiency of lifestyle interventions. A family history of diabetes is known as one of the major risk factors for T2DM [3, 4]. Family history demonstrates the effects of genetic factors, clustered family lifestyle factors, and the relation between the features stated earlier [5]. Family history of diabetes also

influences insulin resistance and insulin secretion, in the Chinese population [6]. The Finnish Diabetes Prevention Study (DPS) found that patients with reduced glucose tolerance and a family history of diabetes had a significant weight loss and decrease in two-hour plasma glucose relative to individuals without a family history of diabetes in the 1st year of lifestyle intervention [7]. Previous reports documented that lifestyle intervention might be much prominent in the descendants of patients with T2DM compared to those without a family history of diabetes, with higher insulin sensitivity index in response to physical activity intervention, representing that insulin sensitivity is extremely controlled by exercise in patients with a family history of T2DM [8, 9].

The purpose of the current study is to evaluate if a patient family history of diabetes is related to the efficiency of lifestyle intervention on insulin resistance and insulin secretion in a cohort of metabolic syndrome with 2-year follow-up.

2. Subjects and Methods

2.1. Study Participants. Study participants were enrolled primarily from the outpatient department of endocrinology of China-Japan Friendship Hospital from June 2010 to May 2011. The selection criteria were as follows: (1) ages 30-70 years; (2) understand the whole process of the program, voluntarily participate, and sign an informed consent form; and (3) central obesity (female: waist circumference > 80 cm; male: waist circumference > 90 cm) accompanied by at least two of the following items:(1) 1.7 mmol/L < TG < 5.6 mmol/L and TC < 7.0 mmol/L; (2) 6.1 mmol/L ≤ fasting blood glucose (FPG) < 7.0 mmol/L; newly diagnosed diabetes mellitus, but FPG should be ≤ 8.0 mmol/L; (3) 130 mmHg ≤ systolic pressure < 160 mmHg, and/or 85 mmHg ≤ diastolic pressure < 100 mmHg, or received antihypertensive therapy; (4) HDL − C < 1.04 mmol/L for men, or <1.29 mmol/L for women. The exclusion criteria were as follows: (1) pregnant women and women who have an intention for pregnancy or breastfeeding; (2) patients who cannot cooperate; (3) patients with diastolic blood pressure ≥ 100 mmHg and/or systolic blood pressure ≥ 160 mmHg; (4) severe heart disease such as frequent angina pectoris or coronary artery bypass grafting or percutaneous coronary intervention, cardiac insufficiency, cardiac enlargement, myocardial infarction, and severe arrhythmia; (5) patients with stroke and transient ischemic attack; (6) patients with abnormal liver function, ALT, and AST more than 2 times of the normal upper limit; (7) patients with impaired renal function: serum creatinine, female ≥ 133 μmol/L, male ≥ 135 μmol/L; (8) patients with chronic gastrointestinal diseases; (9) patients with acute or chronic infections, malignant tumors, mental system diseases, and drug or alcohol addiction; and (10) patients who insist on using weight loss drugs. The exit criteria were as follows: (1) patient withdraws the informed consent form at any stage during the follow-up; (2) unable to cooperate and not following up on time; (3) pregnancy; (4) TG ≥ 5.65 mmol/L, and/or TC ≥ 7.0 mmol/L; (5) diastolic blood pressure > 100 mmHg and/or systolic blood pressure > 160 mmHg; (6) FPG > 11.0 mmol/L; and (7) occurrence of cardiovascular events. Finally, a total of 151 subjects (including 98 women and 53 men) were enrolled. All of the 151 patients finished a 1-year intervention, 124 of them finished a 2-year intervention, and 27 patients dropped out for the loss of follow-up or withdrawing.

2.2. Ethics Statement. The study protocol was approved by the ethics committee of the China-Japan Friendship Hospital (Beijing, China) and conducted in accordance with the Declaration of Helsinki II. We obtained written informed consent from each participant.

2.3. Study Design. A brief information was given to the subjects on how to reach the goals of the intervention: (1) among the patients with BMI (kg/m^2) ≥ 24, decreasing 5–10% of initial body weight, and between patients with BMI < 24, request no weight loss; (2) <30% of energy derived from total fat consumption; (3) 55–65% of energy result from carbohydrate consumption, avoidance/decrease of refined carbohydrates, and white sugars; (4) 20–30 g fiber consumption/day, such as brown rice, whole grains, corn, fruits, and vegetables; and (5) doing modest or forceful physical activity for at least half an hour a day. The participants received monthly face-to-face sessions with study physicians. A detailed program was offered annually; in other monthly sessions, physicians only checked patients' weight, waist circumference and blood pressure, and gave them general oral evidence on the awareness of diabetes, dietary modification, and rising physical activity.

At baseline and at every annual visit, all participants completed a questionnaire about medical history and underwent a medical examination and an oral glucose tolerance examination.

2.4. Definitions. Diabetes was identified according to the 1999 World Health Organization (WHO) criteria of fasting plasma glucose (FPG) ≥ 7.0 mmol/L, 2 − h postprandial plasma glucose (2 − h PPG) ≥ 11.1 mmol/L, or a self-reported history of diabetes. Prediabetes was defined as FPG ≥ 6.1 and < 7.0 mmol/L and/or 2 − h PPG ≥ 7.8 and < 11.1 mmol/L. According to the 2005 IDF consensus worldwide definition of the metabolic syndrome for Asians, the criteria of MetS must include central obesity (waist circumference > 90 cm for males and >80 cm for females), plus two or more of the following risk factors, i.e., low HDL cholesterol (males < 1.04 mmol/L and females < 1.29 mmol/L, or under treatment), high serum triglycerides (>1.7 mmol/L, or under treatment), increased blood pressure (≥130/85 mmHg or under treatment), and fasting blood glucose (≥5.6 mmol/L or under treatment) [10].

2.5. Clinical Information and Laboratory Measurements

2.5.1. Sociodemographic Characteristics. Data were collected with a planned questionnaire through a face-to-face interview to assess general information, personal history, family history, and history of current illness. A family history of diabetes was self-reported by a questionnaire at baseline. For the patients who have even one of first-degree relatives with

Table 1: Baseline clinical characteristics of the participants based on the history of diabetes.

	DMFH- ($n = 90$)	DMFH+ ($n = 61$)	P
Age (year)	48.76 ± 8.95	48.93 ± 7.96	0.905
Male sex (n, %)	32 (35.6)	21 (34.4)	0.887
WC (cm)	92.96 ± 8.11	93.12 ± 9.09	0.908
Weight (kg)	74.67 ± 10.40	74.61 ± 10.03	0.972
WHR	0.90 ± 0.06	0.90 ± 0.05	0.886
Body mass index (kg/m^2)	28.28 ± 3.34	28.34 ± 4.12	0.925
Systolic blood pressure (mmHg)	133.96 ± 12.01	132.05 ± 11.14	0.326
Diastolic blood pressure (mmHg)	86.99 ± 7.16	83.82 ± 10.35	0.028
Total cholesterol (mmol/L)	5.05 ± 1.04	4.84 ± 0.74	0.185
Triglyceride (mmol/L)	2.41 ± 1.23	2.10 ± 0.67	0.083
HDL-C (mmol/L)	1.17 ± 0.26	1.20 ± 0.25	0.554
LDL-C (mmol/L)	3.20 ± 1.12	3.30 ± 0.86	0.579
Fasting glucose (mmol/L)	5.62 ± 0.71	5.63 ± 0.78	0.980
30 min glucose (mmol/L)	9.89 ± 1.94	10.50 ± 2.46	0.091
2-h glucose (mmol/L)	7.70 ± 2.06	7.86 ± 2.88	0.696
Fasting insulin (mU/L)	9.63 ± 6.57	10.43 ± 6.81	0.483
30 min insulin (mU/L)	58.21 ± 39.45	62.13 ± 43.20	0.578
2-h insulin (mU/L)	49.23 ± 43.75	59.89 ± 50.34	0.182
HbA_1C	5.45 ± 0.59	5.58 ± 0.74	0.214

diabetes, the family history of diabetes was measured to be positive.

2.5.2. Anthropometric Measurements. Subjects were examined for hip circumference (HC), weight, height, waist circumference (WC), and blood pressure. Height and body weight were measured by standard protocol. BMI was calculated as weight/height^2 (kg/m^2). The WC was the circumference of the waist at the horizontal line of the umbilicus measured in centimeters through a measuring tape, and the HC was the circumference of hips at the horizontal line of the anterior superior spine measured in centimeters using a measuring tape. The blood pressure values used were an average of three measurements, which were taken 2 min apart using a mercury sphygmomanometer.

2.5.3. Laboratory Examination. Venous blood samples after 8-14 hours of fasting were obtained from subjects for the measurement of triglyceride (TG), fasting blood glucose, HbA1c, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). Blood samples during a 75 g oral glucose tolerance test (OGTT) were collected at 0 min, 30 min, and 2 h. By radioimmunoassay, serum insulin was assessed. In order to test the β-cell function, homeostatic model tests for insulinogenic indices and β-cell function (HOMA-B) were measured [11, 12]. The Matsuda index (ISIm) and HOMA-insulin resistance (IR) index were used to evaluate insulin sensitivity [12, 13].

2.6. Statistical Analysis. Continuous data are described using 95% confidence intervals and means, while categorical variables are concluded using percentages and counts. Continuous variables that were not normally distributed, including HOMA-B, HOMA-IR, ISIm, and insulinogenic indices, were natural log-transformed before analyses. The independent-sample t-test and χ^2-test were used to evaluate the differences between the groups with and without a family history of diabetes for baseline characteristics and changes in characteristics. The paired-samples t-tests were used to evaluate the changes from baseline to 1 year/2 year. The general linear models were used to measure family history-lifestyle interaction effect on changes in primary outcomes, and multivariate-adjusted models were performed including age, gender, baseline BMI, baseline waist circumference, and family history of diabetes at baseline (model 1). Weight loss at 1 year and 2 years based on model 1 (model 2) were further adjusted. All mentioned P values were nominal and 2-sided, and $P < 0.05$ was considered significant. All statistical analyses were performed using the SPSS statistical software version 20.0.

3. Results

3.1. Clinical Characteristics. Among the 151 participants, the DMFH(-) and DMFH(+) groups had 90 and 61 participants. Compared to those in the DMFH(-) group (DBP 86.99 ± 7.16 mmHg), participants in the DMFH(+) (DBP 83.82 ± 10.35 mmHg) group had lower diastolic blood pressure. There were no significant differences among other clinical parameters, as shown in Table 1.

Table 2: One-year glucose tolerance outcome in subjects with and without a family history of diabetes.

		DMFH- ($n = 90$)			DMFH+ ($n = 61$)		
		NGT	Pre-DM	DM	NGT	Pre-DM	DM
Baseline (n, %)		45 (50.0)	36 (40.0)	9 (10.0)	34 (55.7)	17 (27.9)	10 (16.4)
1year outcome	NGT (n, %)	33 (73.3)	15 (41.7)	0 (0.0)	30 (88.2)	8 (47.1)	2 (20.0)
	Pre-DM (n, %)	11 (24.5)	18 (50.0)	2 (22.2)	3 (8.9)	7 (41.1)	5 (50.0)
	DM (n, %)	1 (2.2)	3 (8.3)	7 (77.8)	1 (2.9)	2 (11.8)	3 (30.0)

Table 3: Two-year glucose tolerance outcome in subjects with and without a family history of diabetes.

		DMFH- ($n = 78$)			DMFH+ ($n = 46$)		
		NGT	Pre-DM	DM	NGT	Pre-DM	DM
Baseline (n, %)		40 (51.3)	30 (38.5)	8 (10.2)	25 (54.3)	12 (26.1)	9 (19.6)
2-year outcome	NGT (n, %)	30 (75.0)	14 (46.7)	3 (37.5)	17 (68.0)	7 (58.3)	0 (0.0)
	Pre-DM (n, %)	8 (20.0)	12 (40.0)	3 (37.5)	7 (28.0)	4 (33.3)	5 (55.6)
	DM (n, %)	2 (5.0)	4 (13.3)	2 (25.0)	1 (4.0)	1 (8.4)	4 (44.4)

3.2. Baseline/1-Year/2-Year Glucose Tolerance Characteristics in Subjects with and without Family History of Diabetes. According to the 1998 WHO criteria, the baseline proportion of normal glucose tolerance (NGT), prediabetes, and diabetes was 50.0% (45/9), 40.0% (36/90), and 10.0% (9/90) in the DMFH(-)group, and 55.7% (34/61), 27.9% (17/61), and 16.4% (10/61) in the DMFH(+) group, respectively. After 1-year lifestyle intervention, in the DMFH(-)group, 26.7% of the NGT participants developed into prediabetes or DM, 41.7% of the pre-DM patients turned into NGT, and 8.3% of them developed into DM. 22.2% of the DM patients turned into pre-DM. In the DMFH(+)group, 11.8% of the NGT participants developed into pre-diabetes or DM, 47.1% of the pre-DM patients turned into NGT, and 11.8% of them developed into DM. 50.0% of the DM patients turned into pre-DM, and 20% of them turned into NGT (Table 2). After a 2-year lifestyle intervention, in the DMFH(-) group, 25.0% of the NGT participants developed into pre-diabetes or DM, 46.7% of the pre-DM patients turned into NGT, and 13.3% of them developed into DM. 37.5% of the DM patients turned into pre-DM, and 37.5% of them turned into NGT. In the DMFH(+) group, 32.0% of the NGT participants developed into pre-diabetes or DM, 58.3% of the pre-DM patients turned into NGT, and 8.4% of them developed into DM. 55.6% of the DM patients turned into pre-DM, and none of them turned into NGT (Table 3).

3.3. Changes of Insulin Secretion and Insulin Resistance Indices after 1-Year/2-Year Lifestyle Intervention in Subjects with and without a Family History of Diabetes. Clinical characteristics related to glucose metabolism such as weight, fasting/30 min/2 h glucose, fasting/2 h insulin during OGTT, and indices such as HOMA-B, HOMA-IR, ISIm, and Insulinogenic index were compared separately in and between the two groups throughout the 2-year intervention. All of the 151 participants finished 1-year intervention; the above indices show no significant difference between the two groups at baseline, except for Ln ISIm, and the DMFH(+) group had lower ISIm compared to DMFH(-) (1.48 ± 0.47 vs. 1.68 ± 0.52, $P = 0.045$), which indicates lower insulin sensitivity. At the end of the 1st year, the above parameters have shown no significant difference between the two groups. During the 1st year intervention, Ln Insulinogenic index ($\Delta_{\text{baseline}-1\text{year}} = 0.29 \pm 0.65$, $P = 0.001$) and 30-min glucose ($\Delta_{\text{baseline}-1\text{year}} = -0.41 \pm 1.71$, $P = 0.024$) changed significantly in the DMFH(-) group; in the DMFH(+) group, Ln ISIm ($\Delta_{\text{baseline}-1\text{year}} = -0.22 \pm 0.60$, $P = 0.022$) and 30-min glucose ($\Delta_{\text{baseline}-1\text{year}} = 0.53 \pm 1.89$, $P = 0.032$) changed significantly, and there was no change reported among the other parameters. The change of 30 min glucose during 1-year intervention has shown a significant difference between the two groups ($P = 0.002$) (Table 4).

A total of 124 participants finished the 2-year intervention; at the end of the 2nd year, the DMFH(+) group had lower Ln Homa-B (4.02 ± 0.88 vs. 4.43 ± 1.14, $P = 0.039$) and fasting insulin (7.15 ± 5.54 vs. 11.25 ± 9.99, $P = 0.013$) compared to the DMFH(-) group. During the 2-year intervention, the Ln Insulinogenic index changed significantly in the DMFH(-) group ($\Delta_{\text{baseline}-1\text{year}} = 0.33 \pm 0.66$, $P < 0.001$ and $\Delta_{\text{baseline}-2\text{year}} = 0.43 \pm 1.17$, $P = 0.034$). Fasting insulin ($\Delta_{\text{baseline}-2\text{year}} = 2.95 \pm 8.69$, $P = 0.034$), 2 h insulin ($\Delta_{\text{baseline}-2\text{year}} = 23.75 \pm 44.89$, $P = 0.002$), Ln HOMA-B ($\Delta_{\text{baseline}-2\text{year}} = 0.43 \pm 1.02$, $P = 0.009$), Ln HOMA-IR ($\Delta_{\text{baseline}-2\text{year}} = 0.53 \pm 1.04$, $P = 0.002$), Ln ISIm ($\Delta_{\text{baseline}-2\text{year}} = -0.52 \pm 0.95$, $P = 0.004$), and Ln Insulinogenic index ($\Delta_{\text{baseline}-2\text{year}} = 0.66 \pm 1.18$, $P = 0.047$) changed significantly after 2 years of intervention compared to baseline in the DMFH(+) group. The change of Ln ISIm ($P = 0.023$), fasting ($P = 0.030$), and 2 h insulin ($P = 0.007$) during the 2-year intervention has shown a significant difference between the two groups (Table 5).

Table 4: Baseline characteristics and 1-year changes in insulin secretion and insulin resistance according to the family history of type 2 diabetes.

	DMFH- ($n = 90$)				DMFH+ ($n = 61$)					
	Baseline	1 year	Change	$P_{\Delta\text{baseline}-1\text{year}}$ *	Baseline	1 year	Change	$P_{\Delta\text{baseline}-1\text{year}}$ *	$P_{\Delta\text{DMFH}(-)\text{VS}(+)}$ **	$P_{\text{baseline DMFH}(-)\text{VS}(+)}$
Ln Homa-IR	0.70 ± 0.60	0.56 ± 0.61	0.13 ± 0.66	0.064	0.81 ± 0.58	0.66 ± 0.78	0.14 ± 0.74	0.183	0.986	0.296
Ln Homa-B	4.39 ± 0.73	4.31 ± 0.64	0.10 ± 0.75	0.233	4.46 ± 0.60	4.43 ± 0.70	0.06 ± 0.71	0.533	0.774	0.541
Ln Insulinogenic index	2.40 ± 0.83	2.14 ± 0.74	0.29 ± 0.65	0.001	2.27 ± 0.84	2.30 ± 0.90	0.05 ± 0.90	0.746	0.105	0.428
Ln ISIm	1.68 ± 0.52	1.76 ± 0.60	−0.08 ± 0.51	0.201	1.48 ± 0.47	1.63 ± 0.66	−0.22 ± 0.60	0.022	0.204	0.045
Weight (kg)	74.67 ± 10.40	72.7 ± 10.35	2.30 ± 11.59	0.084	74.61 ± 10.03	74.8 ± 9.01	0.67 ± 6.75	0.507	0.934	0.972
Fasting glucose (mmol/L)	5.62 ± 0.71	5.61 ± 0.89	0.01 ± 0.74	0.867	5.63 ± 0.78	5.56 ± 0.90	0.07 ± 0.82	0.530	0.680	0.980
30 min glucose (mmol/L)	9.89 ± 1.94	10.30 ± 2.11	−0.41 ± 1.71	0.024	10.50 ± 2.46	9.97 ± 2.25	0.53 ± 1.89	0.032	0.002	0.091
2-h glucose (mmol/L)	7.70 ± 2.06	7.95 ± 2.67	−0.25 ± 2.06	0.249	7.86 ± 2.88	7.48 ± 2.69	0.38 ± 2.38	0.219	0.085	0.696
Fasting insulin(mU/L)	9.63 ± 6.57	8.39 ± 5.35	1.27 ± 6.06	0.052	10.43 ± 6.81	10.13 ± 9.52	0.13 ± 7.22	0.897	0.313	0.483
2-h insulin(mU/L)	49.23 ± 43.75	46.09 ± 43.09	3.21 ± 33.78	0.375	59.89 ± 50.34	52.18 ± 45.93	8.28 ± 43.30	0.170	0.439	0.182

HOMA-B: the homeostasis model assessment for β-cell function; HOMA-IR: the homeostasis model assessment for insulin resistance; ISIm: Matsuda index; $P < 0.05$ is considered statistically significant. *P values for paired-samples t-tests for changes from baseline to 1 year. **P values for independent sample t-test for 1-year changes.

Table 5: Baseline characteristics/1-year/2-year changes in insulin secretion and insulin resistance based on the family history of type 2 diabetes.

	DMFH- (n =78)							DMFH+ (n = 46)									
	Baseline	1 year	2 years	change$_{\Delta baseline-1year}$	change$_{\Delta baseline-2year}$	$P_{\Delta baseline-1year*}$	$P_{\Delta baseline-2year*}$	Baseline	1 year	2 years	change$_{\Delta baseline-1year}$	change$_{\Delta baseline-2year}$	$P_{\Delta baseline-1year*}$	$P_{\Delta baseline-2year*}$	$P_{1year\Delta DMFH(-)VS(+)**}$	$P_{2year\Delta DMFH(-)VS(+)**}$	$P_{baseline\,DMFH(-)VS(+)}$
Ln Homa-IR	0.69 ± 0.61	0.54 ± 0.63	0.58 ± 0.98	0.15 ± 0.69	0.13 ± 1.18	0.058	0.354	0.78 ± 0.61	0.69 ± 0.81	0.26 ± 0.87	0.06 ± 0.76	0.53 ± 1.04	0.595	0.002	0.524	0.070	0.449
Ln Homa-B	4.40 ± 0.75	4.30 ± 0.67	4.43 ± 1.14	0.13 ± 0.78	−0.01 ± 1.32	0.160	0.959	4.41 ± 0.56	4.40 ± 0.72	4.02 ± 0.88	0.02 ± 0.76	0.43 ± 1.02	0.836	0.009	0.500	0.065	0.941
Ln Insulinogenic index	2.39 ± 0.87	2.10 ± 0.75	1.98 ± 1.18	0.33 ± 0.66	0.43 ± 1.17	0.000	0.034	2.26 ± 0.91	2.22 ± 0.89	1.97 ± 0.98	0.10 ± 0.97	0.66 ± 1.18	0.575	0.047	0.181	0.521	0.513
Ln ISIm	1.70 ± 0.52	1.79 ± 0.61	1.78 ± 0.86	−0.11 ± 0.54	−0.04 ± 0.92	0.149	0.786	1.50 ± 0.45	1.62 ± 0.63	2.08 ± 0.80	−0.16 ± 0.59	−0.52 ± 0.95	0.130	0.004	0.650	0.023	0.075
Weight (kg)	75.10 ± 10.59	74.92 ± 11.21	72.80 ± 10.38	0.18 ± 7.05	2.24 ± 11.65	0.820	0.096	75.18 ± 9.24	75.60 ± 8.45	74.91 ± 9.08	−0.12 ± 4.80	0.73 ± 6.82	0.870	0.479	0.800	0.436	0.965
Fasting glucose (mmol/L)	5.59 ± 0.73	5.59 ± 0.90	5.45 ± 0.82	0.00 ± 0.77	0.13 ± 0.9	0.988	0.192	5.65 ± 0.80	5.64 ± 0.94	5.55 ± 0.85	0.01 ± 0.85	0.10 ± 0.86	0.932	0.430	0.950	0.845	0.647
30 min glucose (mmol/L)	9.92 ± 2.02	10.27 ± 2.13	9.88 ± 2.21	−0.35 ± 1.75	0.05 ± 2.32	0.080	0.858	10.56 ± 2.67	10.12 ± 2.39	10.01 ± 2.29	0.43 ± 1.86	0.55 ± 2.07	0.120	0.078	0.020	0.230	0.136
2-h glucose (mmol/L)	7.71 ± 2.00	8.02 ± 2.74	7.56 ± 2.11	−0.30 ± 2.15	0.13 ± 2.28	0.216	0.604	8.09 ± 3.00	7.61 ± 2.76	7.94 ± 2.82	0.48 ± 2.52	0.15 ± 2.42	0.207	0.675	0.070	0.969	0.407
Fasting insulin (mU/L)	9.66 ± 6.86	8.32 ± 5.58	11.25 ± 9.99	1.39 ± 6.37	−1.60 ± 11.71	0.058	0.239	10.11 ± 6.55	10.48 ± 10.6	7.15 ± 5.54	−0.69 ± 7.21	2.95 ± 8.69	0.545	0.034	0.109	0.030	0.730
2-h insulin (mU/L)	48.28 ± 43.63	45.76 ± 44.13	48.88 ± 43.31	2.55 ± 33.45	−0.46 ± 46.21	0.502	0.932	50.04 ± 43.3	50.59 ± 44.95	36.67 ± 40.45	6.53 ± 41.73	23.75 ± 44.89	0.329	0.002	0.576	0.007	0.419

HOMA-B: the homeostasis model assessment for β-cell function; HOMA-IR: the homeostasis model assessment for insulin resistance; ISIm: Matsuda index. $P < 0.05$ is considered statistically significant. $^{*}P$ values for paired-samples t-tests for changes from baseline to 1 year/2 years. $^{**}P$ values for independent sample t-test for 1-year/2-year changes.

Table 6: Effects of the family history of diabetes on changes in insulin secretion and insulin resistance in the response of lifestyle intervention.

	1-year outcome			2-year outcome		
	β	SE	P	β	SE	P
Model 1						
ΔHOMA-B	0.030	0.129	0.819	-0.406	0.238	0.091
ΔHOMA-IR	-0.013	0.123	0.918	-0.361	0.222	0.107
ΔInsulinogenic index	0.279	0.154	0.077	-0.244	0.342	0.479
ΔISIm	0.148	0.112	0.190	0.500	0.210	0.020
Model 2						
ΔHOMA-B	0.030	0.130	0.816	-0.399	0.243	0.104
ΔHOMA-IR	-0.110	0.115	0.340	-0.344	0.225	0.130
ΔInsulinogenic index	0.276	0.155	0.078	-0.230	0.348	0.512
ΔISIm	0.150	0.112	0.182	0.476	0.211	0.027

Adjusting for age, baseline BMI, sex, and baseline waist circumference in model 1 and with extra adjustment for weight change in model 2.

3.4. Associations of Family History of Diabetes with the Insulin Secretion-Related and Insulin Resistance-Related Indices after 1 or 2 Years of Lifestyle Intervention. The relationship between family history of diabetes and 1-year changes in HOMA-IR, HOMA-B, ISIm, or Insulinogenic index was not significantly changed by lifestyle intervention adjusting for age, baseline BMI, sex, baseline waist circumference in model 1, and with extra tuning for weight change in model 2. A family history of diabetes was related to a 0.500 unit increase in the 2-year ISIm ($P = 0.020$) in model 1 and a 0.476 unit increase in the 2-year ISIm ($P = 0.027$) in model 2. However, there was no significant association between family history of diabetes and 2-year changes in HOMA-IR, HOMA-B, or Insulinogenic index (Table 6).

4. Discussion

Nutrient excess and sedentary behaviors of our modern society are indications of metabolic syndrome, which significantly increases T2DM risk and gives a natural state of decreased insulin sensitivity and offers a common physiological β-cell challenge. Lifestyle intervention reports have shown that a diet/exercise regimen reduces IGT progression to T2DM by increasing insulin sensitivity and increasing insulin secretion. Lifestyle management is also the basis for the medical care of diabetes[14, 15]. In our study, we enrolled MetS patients with normal glucose, prediabetes, or mild newly onset diabetes and performed a 2-year lifestyle intervention to examine its effects on insulin secretion and insulin resistance.

Based on our 2-year longitudinal study, the results indicated that patients with a family of diabetes and metabolic syndrome would benefit more significantly from lifestyle intervention in regard to insulin resistance compared to those without a family history and not dependent on weight change. In patients without a family history of diabetes, the Insulinogenic index which indicated an early phase of insulin secretion continued to worsen throughout 2 years of lifestyle intervention; however, insulin resistance did not change significantly in the DMFH(-) group. This result indicated that in these patients, besides the traditional lifestyle intervention plan, the additional targeted method should be applied to ameliorate the early-phase insulin secretion, such as low glycemic index food, or α-glucosidase inhibitor. It is worthy to note that the discrepancy in insulin secretion and insulin resistance between different races, Asians appear to worse in early phase insulin secretion than Caucasians [16]. A previous study shown that T2DM in East Asians was characterized primarily by β-cell dysfunction, which was evident immediately after ingestion of glucose or meal, and less adiposity compared to that in Caucasians [17]. A study has shown that increased HbA_1c was related to compromised early-phase insulin secretion relatively compared to the insulin resistance in Koreans which were at high risk of diabetes [18]. In patients with a family history of diabetes, the delightful change in 1 year was an improvement of ISIm, and this continued in the 2nd year with improved HOMA-IR; however, HOMA-B and Insulinogenic index, which represented insulin secretion, worsen in the 2nd year.

In the present study, intervention in lifestyle involved dietary improvements (intake of calories, fat, carbohydrates, fiber) and physical activity increases. Convincing research indicated that dietary variables controlled the sensitivity of insulin [19, 20]. Cohort and nutritional intervention studies suggested that people with greater genetic predisposition could prevent complex dietary habits which were more detrimental in the heterogeneity of a particular T2DM-related phenotype [21]. Furthermore, enriched exercise could also advance insulin sensitivity and glucose homeostasis [22]. In summary, we concluded that the combination of enhanced dietary intakes and physical activity could alter the impact of the diabetes family history on insulin sensitivity.

Family history not only represents the ramifications of numerous genetic variables, but also the family's clustered lifestyle variables. A previous study has shown that, despite having comparable recorded physical activities and exercise to that of individuals with no family history, a family history of T2DM was correlated with lower physical fitness. The same participants have reduced insulin secretion optimized for insulin resistance, despite increased BMI [23]. A previous study has shown that individuals without a family history of diabetes were more effective in reacting to lifestyle therapy

about cardiometabolic assessments and glucose tolerance compared to the individuals with a family history of diabetes in a 1-year follow-up in a cohort of Finnish men at significant risk for T2DM [24]. However, in our study, the patients of the two groups have shown no significant difference in fasting glucose, 2 h glucose, or HbA_1c, and those with a family history of diabetes were more effective in retorting to lifestyle therapy in terms of insulin resistance.

The family history-lifestyle intervention on changes in insulin resistance only becomes significant in 2 years. These findings were different from the findings reported in other long-term diet or lifestyle intervention trials such as the A TO Z Weight Loss Study and the DIRECT trial [25, 26]. In the A TO Z Weight Loss Study and DIRECT trial, the changes in insulin resistance weakened from 1 year to 2 years, comparatively due to reduced devotion to exercise and dietary and intervention after 1 year. However, in our study, both groups did not change significantly in body weight, especially in the DMFH(+) group, and the result has shown family history affected the ISIm after adjusting baseline BMI and weight change.

The current study has many significant findings. The present study was a 2-year longitudinal study, the findings were based on longitudinal measures of weight and glycemic markers, and its participants were suffering from metabolic syndrome including NGT, prediabetes, and diabetes. The most important and common feature was insulin resistance. We performed OGTT to get indices such as ISIm and Insulinogenic index in addition to HOMA-B and HOMA-IR, which were only based on fasting glucose and insulin to get a more comprehensive profile of β-cell function. At the same time, the present study gives insight into the various mechanisms that support family history affecting the effectiveness of lifestyle intervention.

This study also has limitations. First, at subsequent monthly visits, all the participants received written and general oral guidance on diabetes awareness, dietary change, and increased exercise, although no unique individualized programs were provided. We did not perform a questionnaire on the changes in the main exercise habits and dietary, 3-day 24-h food records to testify the result, and the level of our intervention. This kind of lifestyle intervention saved time and mimicked the most common lifestyle intervention in the clinical practice of outpatient departments in China, but it made the procedure less precise and targeted. Secondly, the comparatively small sample size may restrict the power of the analysis to detect much more moderate associations.

In conclusion, we reported a family history of diabetes was related with a 0.500 unit greater increase in 2-year ISIm ($P = 0.020$), modified by lifestyle intervention adjusting for age, baseline BMI, sex, and baseline waist circumference, and a 0.476 unit greater increase in 2-year ISIm ($P = 0.027$) with extra adjustment for weight change. These findings indicated that patients with a family history of diabetes were more effective in acting to lifestyle counselling with regard to insulin resistance than those without a family history of diabetes with additional adjustment for weight change. In this regard, it stresses the significance of a thorough examination of family history, in the risk assessment and development of more targeted therapeutic strategies for lifestyle interventions and diabetes prevention.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

All authors contributed significantly and met the criteria of authorship.

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Research Article

Association of Apolipoprotein E Polymorphisms and Risks of Ischemic Stroke in Chinese Patients with Type 2 Diabetes Mellitus

Na Wang,[1] **Qian Liu**,[2] **Hui Liu**,[2] **Xiao Cong**,[2] **Hui Yang**,[2] **Yang Yu**,[2] **Yongtong Cao**,[1,2] **and Liang Ma**[2]

[1]*Department of Transfusion Medicine, China-Japan Friendship Hospital, Beijing 100029, China*
[2]*Clinical Laboratory, China-Japan Friendship Hospital, Beijing 100029, China*

Correspondence should be addressed to Yongtong Cao; caoyongtong92@sina.com and Liang Ma; liangma321@aliyun.com

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Background. The apolipoprotein E (*APOE*) gene polymorphisms have been intensively studied in patients with type 2 diabetes mellitus (T2DM) and ischemic stroke (IS) in recent years. However, it is unclear whether *APOE* gene polymorphisms are correlated with increased risk for developing IS in T2DM patients. Thus, this study was designed to examine the association between *APOE* gene polymorphisms and risks of IS in Chinese patients with T2DM. *Methods*. This case-control study enrolled 243 subjects with T2DM as controls, and 210 subjects with T2DM complicated with IS as case patients. The genotypes were determined using real-time PCR while HbA1c and lipid levels were detected using commercially available kits. *Results*. The systolic blood pressure (SBP), diastolic blood pressure (DBP), and the proportion of patients with a history of hypertension were higher in the case patients than that in the controls. We confirmed that the ε2/ε3 genotype, as well as SBP and history of hypertension, was the independent risk factor for developing IS in T2DM patients. *Conclusions*. We conclude that the ε2/ε3 genotype might contribute to the increased risk for developing IS in Chinese patients with T2DM.

1. Introduction

China has the highest number of diabetes in the world. According to the International Diabetes Federation (IDF) report, there were 116 million Chinese diagnosed with diabetes in 2019, and the number will increase to 141 million by 2030 [1]. Ischemic stroke (IS) is a cerebrovascular complication of type 2 diabetes mellitus (T2DM) due to accelerated atherosclerosis and carotid artery disease development [2]. Both macrovascular and microvascular systems of the brain are severely affected in T2DM [3]. T2DM exacerbates ischemic brain injury and worsens functional outcome after stroke [4]. Patients with T2DM have a two- to four-fold increase in the risk of IS compared with the general population [5, 6]. Moreover, stroke patients with diabetes have a poor prognosis after stroke onset [7]. Thus, it is of great importance to investigate the risk factors of T2DM patients complicated with IS.

Mounting evidence indicates that the apolipoprotein E (*APOE*) gene located at chromosome 19q13.32 is a candidate gene in the development of T2DM and IS [8–10]. The protein encoded by the *APOE* gene consists of 299 amino acid residues and contains amphipathic α-helical lipid-binding structural domains which enable APOE to interact with members of the low-density lipoprotein receptor family. Through the interaction, APOE plays an essential role in lipid transportation in both plasma and brain [11]. The dysregulation of the *APOE* expression and genetic variance of the *APOE* influence the APOE functions and lead to the pathogenesis of nervous and cardiovascular diseases eventually [11–13]. The common gene variants, epsilon-2 (ε2), epsilon-3 (ε3), and epsilon-4 (ε4), are generated by the two single-nucleotide polymorphisms (SNPs) rs7412 (C/T) and rs429358 (C/T) in exon 4 of the *APOE* gene. The variants are different haplotypes of the *APOE* gene generated by the combination of the two SNPs at the *APOE* locus [14]. The most common allele iso-

form is ε3 with a frequency of 70-80%. The ε2 and ε4 have a frequency of 5-10% and 10-15%, respectively [15]. The 3 alleles of the *APOE* gene form 6 genotypes (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4) [16]. The ε3/ε3 is the most common genotype with a frequency of approximately 67% [8]. The frequencies of ε3/ε4 and ε2/ε3 are lower than those of ε3/ε3, and ε2/ε2, ε4/ε4, and ε2/ε4 genotypes have the lowest frequencies [17].

Understanding the risk factors of T2DM patients complicated with IS may shed light into intervening the development of complications of T2DM. So far, studies on the association of *APOE* polymorphisms and IS risks in Chinese patients with T2DM are lacking. Thus, this study is designed to elucidate whether the *APOE* gene polymorphism is an essential determinant of Chinese patients with T2DM complicated with IS.

2. Materials and Methods

2.1. Subjects. A case-control study was carried out from July 2015 to July 2018. Informed consent was obtained from all enrolled subjects, and the design protocol was approved by the Ethics Committee of China-Japan Friendship Hospital. The data of the families, their medical history, and smoking habits of the patients was obtained by a questionnaire. Clinical examination including measurement of systolic blood pressure (SBP) and diastolic blood pressure (DBP) was applied. Anthropometric data (weight and height) were collected and used for BMI calculation. Hypertension was defined as blood pressure above 140/90 mmHg or taking antihypertensive drugs. Dyslipidemia was characterized by increased total cholesterol (TC ≥ 6.20 mmol/L), low-density lipoprotein cholesterol (LDL − C > 4.13 mmol/L), and triglyceride (TG > 2.25 mmol/L), or decreased high-density lipoprotein cholesterol (HDL − C < 1.03 mmol/L) [18]. These subjects were categorized into two groups: T2DM patients complicated with and without IS according to the criteria of the American Diabetes Association Classification 2010 [19].

(1) *T2DM patients without IS (control group).* This group consists of 243 subjects fulfilling the T2DM diagnostic criteria or under diabetes medication (oral and/or insulin) with no history or signs of any IS.

(2) *T2DM patients complicated with IS (IS group).* This group consists of 210 subjects diagnosed to have T2DM or under diabetes medication and complicated with IS. All the subjects in this group were examined by a qualified neurologist. The diagnosis of IS was confirmed by clinical symptoms or signs, laboratory results, and computed tomography (CT) or magnetic resonance imaging (MRI). Exclusion criteria included cardiac diseases, renal diseases, hepatic diseases, endocrine diseases, metabolic disorders, autoimmune diseases, skeletal disorders, and cancerous diseases.

2.2. DNA Isolation and APOE Genotyping. Blood samples were collected in vacuum tubes and stored at -20°C until processed. DNA was extracted from each blood sample using the genomic DNA purification kit (Xi'an Tianlong Science and Technology Co., Ltd., Xi'an, Shaanxi, China). The concentration of DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Three *APOE* alleles (ε2, ε3, and ε4) were detected by an *APOE* Genotyping Kit (Sinochips Bioscience Co., Ltd., Zhuhai, Guangdong, China). Polymorphic alleles were identified by the fluorescence intensity of the hybridization sites.

2.3. Biochemical Analysis. Plasma levels of fasting TG, TC, LDL-C, and HDL-C were measured using the AU5800 automated biochemical analyzer (Beckman Coulter, Brea, CA, USA). HbA1c was detected using the D-10 Hemoglobin Testing System (Bio-Rad, Hercules, CA, USA).

2.4. Statistical Analysis. The data were statistically analyzed using SPSS version 24.0 software (IBM, Chicago, IL, USA). Quantitative data were expressed as mean values ± standard deviation (SD). Normally distributed data were compared using Student's *t*-test. The significance of differences in the proportion of patients with a history of hypertension between the two groups was tested by the chi-square test (χ^2). The differences in the distribution frequencies of genotype and allele between the two groups, and the deviations from Hardy-Weinberg equilibrium were tested by the chi-square test. The extremely rare genotype groups—ε2/ε2, ε2/ε4, and ε4/ε4—were excluded from the genotype and allele analyses. Univariable logistic regression analysis was used to test the association between IS and *APOE* gene polymorphisms, and the analysis results were presented as unadjusted odds ratios (OR) with confidence intervals (95% CI). Multivariate logistic regression was used to determine the risk factors for developing IS in T2DM patients with adjustment for potential covariates: age, gender, BMI, blood pressure, duration of T2DM, smoking, HbA1c level, and plasma lipids, and the results were presented as adjusted ORs. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. General Characteristics and Biochemical Variables of the Patients. 210 T2DM patients complicated with IS and 243 age- and sex-matched controls from the same demographic area were included in this study. Table 1 presents the general characteristics and biochemical variables of the patients in the control and the IS groups. No statistically significant differences were observed between the groups in age, gender, BMI, smoking, diabetes duration, HbA1c level, and plasma lipid level ($P > 0.05$). However, SBP, DBP, and the proportion of patients with a history of hypertension were higher in the IS group than in the control group ($P < 0.05$).

3.2. APOE Genotype and Allele Distribution Frequencies in the IS and the Control Groups. The *APOE* genotype distribution in the IS and the control groups were in Hardy-Weinberg equilibrium ($P > 0.05$). Distribution frequencies of the 6 genotypes and 3 alleles of *APOE* in the IS and the control groups are summarized in Table 2.

Table 1: Demographic, clinical, and biochemical data of the IS and the control groups.

	IS ($n = 210$)	Control ($n = 243$)	P value
Age (years)	65.41 ± 8.20	64.58 ± 10.21	0.338
Gender (male %)	47.62	52.26	0.324
BMI (kg/mm^2)	25.29 ± 3.98	25.75 ± 3.19	0.175
SBP (mmHg)	142.67 ± 19.41	133.98 ± 18.63	<0.001
DBP (mmHg)	81.64 ± 10.17	78.17 ± 10.67	<0.001
Hypertension (%)	84.28	70.20	<0.001
Smokers (%)	18.57	17.70	0.809
Diabetes duration (years)	14.44 ± 6.09	15.10 ± 8.32	0.332
IS duration (years)	3.65 ± 2.10	—	—
HbA1c (%)	7.66 ± 1.72	7.96 ± 1.88	0.084
HbA1c (mmol/mol)	60.25 ± 18.81	63.45 ± 20.59	0.084
TG (mmol/L)	1.88 ± 1.62	2.00 ± 1.46	0.433
TC (mmol/L)	4.20 ± 1.24	4.24 ± 1.14	0.697
LDL-C (mmol/L)	2.48 ± 0.95	2.52 ± 0.99	0.666
HDL-C (mmol/L)	1.05 ± 0.33	1.04 ± 0.31	0.880
Dyslipidemia (%)	64.29	64.20	0.984

3.3. Association of APOE Gene Polymorphisms and IS. The univariate analysis was used to evaluate the association from the perspective of the genotype and allele frequencies of *APOE* gene polymorphisms. The genotype of ε2/ε3 increased the risk of IS in T2DM patients, with unadjusted OR 1.901 (95% CI 1.117-3.297,$P = 0.0179$), while the genotype of ε3/ε4 showed no association with IS in T2DM patients, with unadjusted OR 1.629 (95% CI 0.943-2.756,$P = 0.0694$) (Table 3). The results displayed no significant difference in *APOE* gene allele frequencies between the two groups (Table 3). After being adjusted for age, gender, BMI, SBP, DBP, history of hypertension, smoking habits, diabetes duration, HbA1c, TG, TC, LDL-C, HDL-C, and history of dyslipidemia using multivariable binary logistic regression analysis as shown in Table 4, the ε2/ε3 genotype was an independent risk factor for developing IS in T2DM patients, with adjusted OR 2.225 (95% CI 1.244-3.980, $P = 0.007$). However, the genotype of ε3/ε4 was not found to be an independent risk factor for developing IS in T2DM patients, with adjusted OR 1.727 (95% CI 0.978-3.049, $P = 0.060$) (Table 4). SBP (95% CI 1.004-1.031, $P = 0.013$) and history of hypertension (95% CI 1.089-3.045, $P = 0.022$) were also independent risk factors for developing IS in T2DM patients (Table 4).

4. Discussion

Many studies showed that patients suffering from T2DM are at a higher risk for IS than individuals without T2DM [4, 20]. Thus, it is essential to explore the predisposing risk factors for IS among T2DM patients. We carried out a case-control study to investigate the association of the *APOE* gene polymorphisms and risks of IS in Chinese patients with T2DM. We observed that SBP, DBP, and the proportion of patients with a history of hypertension were higher in the IS group than in the control group. We also found that the ε2/ε3 genotype was an independent risk factor for developing IS in T2DM patients. SBP and history of hypertension were also independent risk factors for developing IS in T2DM patients after adjusting for age, gender, BMI, SBP, DBP, history of hypertension, smoking habits, diabetes duration, HbA1c, TG, TC, LDL-C, HDL-C, and history of dyslipidemia.

A prospective population-based study, with approximately 20-year follow-up, evaluated the effect of T2DM on cardiovascular disease in 13105 samples. The study revealed an increased relative risk for developing stroke of 1.5 to 6.5 fold in T2DM individuals [20]. The increased risk is seen even after early diagnosis. It is reported that the risk of stroke in newly treated patients with T2DM is 9.1% within the first 5 years. The stroke rate is double that of the general population [21]. Many studies have indicated that T2DM patients have residual neurological deficits and a worse functional outcome, along with worse long-term mortality [7, 22]. Therefore, patients and physicians need to aggressively control cardiovascular risk factors soon after T2DM diagnosis. The identification of susceptibility genes and other risk factors would be helpful for the management of IS in T2DM patients.

APOE gene is one of the most widely studied candidate genes of T2DM. In a case-control study recruiting of 451 Thais, it has been demonstrated that ε4 allele containing genotypes were the predictors of T2DM [23]. A meta-analysis of 30 studies including 5423 case patients and 8197 controls suggests that ε2 allele is associated with increased risk of T2DM [8]. Besides, the relation between *APOE* gene polymorphisms and IS has been intensively investigated in many studies. Most findings suggest that ε4 allele increases the odds of IS [24–26]. However, little is known about the link between *APOE* gene polymorphisms and developing IS in T2DM patients. So far, there is only one study conducted on the issue. The study displayed that *APOE* gene polymorphisms were not linked with IS in T2DM patients [27]. However, we found that T2DM patients who carried the ε2/ε3 genotype were at 1.90-fold increased risk to develop IS in the present study. After adjustment for other established risk factors, ε2/ε3 was an independent risk factor for developing IS in T2DM patients. The discrepancies of the findings may be due to ethnic differences, sample sizes, genotyping method, and other risk factors. *APOE* gene polymorphisms affect plasma lipid concentration and may be responsible for the development of IS onset in T2DM patients. Further investigation is needed to study the *APOE* gene polymorphisms and lipid metabolism in T2DM patients complicated with IS.

Consistent with our results, many studies have shown that T2DM patients with hypertension tend to have a higher risk of getting IS [2, 28, 29]. We found that SBP, DBP, and the proportion of patients with a history of hypertension were higher in the IS group compared with that in the control group. Furthermore, SBP and history of hypertension were independent risk factors for developing IS in T2DM patients after adjusting for many related factors. So, proper blood

Table 2: Distribution frequencies of *APOE* genotype and allele in the IS and the control groups.

	APOE genotypes (%)						*APOE* alleles (%)		
	ε2/ε2	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4	ε2	ε3	ε4
IS	2 (0.95)	39 (18.57)	4 (1.90)	126 (60.00)	37 (17.62)	2 (0.95)	47 (11.19)	328 (78.10)	45 (10.71)
Control	45 (1.65)	28 (11.52)	7 (2.88)	172 (70.78)	31 (12.76)	1 (0.41)	43 (8.85)	403 (82.92)	40 (8.23)

Table 3: Associations of *APOE* gene polymorphisms and the risk of IS in T2DM patients compared to that in case patients, represented as unadjusted OR.

	Unadjusted OR (95% CI)	*P* value
Genotype		
ε2/ε3 *vs.* ε3/ε3	1.901 (1.117-3.297)	0.0179
ε2/ε3 *vs.* ε3/ε4	1.167 (0.602-2.281)	0.657
ε3/ε4 *vs.* ε3/ε3	1.629 (0.943-2.756)	0.0694
Allele		
ε2 *vs.* ε3	1.343 (0.876-2.073)	0.186
ε2 *vs.* ε4	0.972 (0.534-1.766)	0.924
ε4 *vs.* ε3	1.382 (0.882-2.190)	0.157

The extremely rare genotypes—ε2/ε2, ε2/ε4, and ε4/ε4 subjects—were excluded from statistical analysis.

Table 4: Associations of *APOE* gene polymorphisms and the risk of IS in T2DM patients compared to that in case patients, represented as adjusted OR.

	Adjusted OR (95% CI)	*P* value
ε3/ε3 (referee)	—	0.010
ε2/ε3	2.225 (1.244-3.980)	0.007
ε3/ε4	1.727 (0.978-3.049)	0.060
Age	1.009 (0.986-1.033)	0.452
Gender	0.880 (0.570-1.358)	0.563
BMI (kg/mm^2)	0.958 (0.901-1.018)	0.166
SBP (mmHg)	1.017 (1.004-1.031)	0.013
DBP (mmHg)	1.017 (0.993-1.042)	0.168
Hypertension (%)	1.821 (1.089-3.045)	0.022
Smokers (%)	1.172 (0.672-2.042)	0.576
Diabetes duration	0.981 (0.954-1.009)	0.186
HbA1c	0.896 (0.798-1.006)	0.063
TG	0.925 (0.772-1.109)	0.398
TC	1.032 (0.580-1.835)	0.916
LDL-C	0.921 (0.496-1.710)	0.793
HDL-C	0.988 (0.387-2.517)	0.979
Dyslipidemia	1.458 (0.841-2.529)	0.179

pressure management is a core issue for mitigating the development of IS in T2DM patients.

There are some limitations in our study which require further investigation. Firstly, the replicability of the data needs to be verified in a larger sample size involving different ethnic groups. Secondly, our study is inadequate in proving the causal relationship but merely an association research due to the study design of case-control. However, our findings indeed contribute to risk prediction for IS in T2DM patients. The genotype ε2/ε3 of *APOE* is possibly a genetic predisposition factor for IS in T2DM patients. Furthermore, it is of great significance to control the blood pressure for T2DM patients to avoid IS onset.

5. Conclusions

Our study indicates that the *APOE* gene polymorphisms are associated with the development of IS in T2DM patients. We also identify ε2/ε3 genotype, SBP, and history of hypertension as independent risk factors in the development of IS in T2DM patients.

This study provides data that may help to improve the ability to identify diabetic individuals at increased risk for IS and improve the clinical management of patients with T2DM.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declared no conflict of interest.

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Research Article

Association between LXR-α and ABCA1 Gene Polymorphisms and the Risk of Diabetic Kidney Disease in Patients with Type 2 Diabetes Mellitus in a Chinese Han Population

Peng Liu[1], **Liang Ma**[2], **Hailing Zhao**[3], **Zhengri Shen**[1], **Xuefeng Zhou**[3], **Meihua Yan**[3], **Tingting Zhao**[3], **Haojun Zhang**[3], **Xinping Qiu**[1] and **Ping Li**[3]

[1]*Shunyi Hospital, Beijing Traditional Chinese Medicine Hospital, Beijing, China*
[2]*Clinical Laboratory, China-Japan Friendship Hospital, Beijing, China*
[3]*Beijing Key Lab for Immune-Mediated Inflammatory Diseases, Institute of Clinical Medical Science, China-Japan Friendship Hospital, Beijing, China*

Correspondence should be addressed to Ping Li; lp8675@163.com

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We designed a case-control study and selected *LXR-α* rs7120118 C>T and *ABCA1* rs2230806 A>G polymorphisms to determine the correlation between these polymorphisms and diabetic kidney disease (DKD) susceptibility in a Chinese Han population. Three hundred DKD patients and 346 type 2 diabetes mellitus (DM) patients without kidney disease were recruited. Our results showed that rs7120118 was associated with DKD (genotype, $P = .027$; allele, $P < .011$). rs7120118 was associated with a higher risk of DKD under a dominant model adjustment by age and sex ($P = .015$) and an additive model ($P = .040$); rs2230806 was associated with a higher risk of DKD under an recessive model ($P < .03$); the combined effect of rs7120118 CC+rs2230806 GG genotype showed an association of DKD adjustment for age and sex ($P = .009$). In subgroup analysis of patients without hypercholesterolemia, the rs2230806 genotype frequencies were different between the two groups ($P = .042$). rs2230806 was associated with increased risk of DKD under a recessive model adjustment for age and sex ($P = .013$) and an additive model ($P = .031$). Our results suggest that *LXR-α* rs7120118 is significantly associated with a higher risk of DKD, and *ABCA1* rs2230806 is significantly associated with a higher risk of DKD without hypercholesterolemia in Chinese Han individuals.

1. Introduction

Diabetic kidney disease (DKD), a devastating complication of diabetes mellitus (DM), is the most common cause of end-stage renal disease (ESRD) and renal failure in the world [1]. According to the International Diabetes Federation, in 2017, there were an estimated 425 million cases of adult DM worldwide, with more than 30% of these cases reported in China [2]. Genetic factors are directly related to the initiation and progression of DKD, including aggregation in families and variable incidence rates between different races [3, 4]. Hence, it is imperative to identify the potentially susceptible genetic *loci* of DKD in the prediction and prevention of DKD [5].

Lipid metabolism disorders are an important factor that leads to DKD progression [6]. As metabolite-sensing receptors, liver X receptors (LXRs) exist in two isoforms: LXR-α (encoded by the NR1H3 gene) and LXR-β (encoded by the NR1H2 gene) [7]. Physiologic processes that are affected by LXRs include inflammation, metabolism and homeostasis of lipids, and cholesterol homeostasis [8]. LXRα could upregulate the expression of hepatic lipogenic enzymes and increase blood TG levels, and LXR-α-deficient mice display markedly prevented hepatic fatty acid synthesis and triglycerides [9]. Multiple common single-nucleotide polymorphisms (SNPs) in LXR-α are associated with a higher risk of coronary heart disease and hemodialysis [10, 11]. Patients carrying the allele T (i.e., CT or TT) at rs7120118 have low

serum lipid levels, while those with the C allele have high serum lipid levels in coronary heart disease and hemodialysis [10, 11]. Furthermore, it has been shown that rs7120118 is associated with the expression of LXR-α [12].

LXR-α, which function as the core determinants of cellular cholesterol homeostasis, induce expression of the cholesterol efflux transporter ATP-binding cassette subfamily A member 1 (ABCA1) to promote cellular cholesterol efflux [13]. As the most prominent member of the ATP-binding cassette family, ABCA1 is highly important for mediating cholesterol efflux from cells [14]. Pedigo et al. found that patients with DKD had decreased expression of ABCA1 impaired cholesterol efflux in macrophages and podocytes [15]. Ganda et al. showed that increased ABCA1-mediated cholesterol efflux evoked tubular cholesterol accumulation in patients with DKD [16]. ABCA1 is involved in high-density lipoprotein cholesterol (HDL-C) biogenesis, and changes in ABCA1 structure and/or protein expression could alter metabolic disturbances [17]. The loci of the ABCA1 gene rs2230806 is located in the major extracellular rings of the ABCA1 protein, which have an important role in APO-I and cholesterol efflux [18]. rs2230806 is also the most widely studied common missense polymorphism, and studies in Asians reported that *ABCA1* SNP (rs2230806, also known as R219K or G1051A) is associated with type 2 DM and coronary heart disease [19, 20].

However, there are limited studies regarding the susceptibility of LXR-α and ABCA1 polymorphisms in Chinese DKD populations. Therefore, because of the heterogeneity for gene polymorphisms in Han Chinese, we tested the hypothesis that there is an association of *LXR-α* rs7120118 (C>T) and *ABCA1* rs2230806 (A>G) with DKD in this population.

2. Materials and Methods

2.1. Patients. This was a case-control study that consisted of 646 volunteers. Three hundred participants had a history of type 2 DM and DKD. They were compared with 346 participants diagnosed with type 2 DM for at least 7 years and had no history of DKD. All participants were recruited from the China-Japan Friendship Hospital (Beijing, China). The study took place from February 2015 to October 2018. Diagnosis of type 2 diabetes mellitus was based on the World Health Organization 1999 criteria [21]. Criteria for DKD were defined by the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF K-DOQI) guidelines [22]. This study was approved by the institutional ethics committee of the China-Japan Friendship Hospital, and informed written consent was obtained from all participants.

2.2. Data Collection, DNA Isolation, and Genotyping. Clinical characteristics of age, sex, body mass index (BMI), duration of diabetes, blood pressure, serum creatinine (Scr), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and HDL-C of each participant were obtained from the medical records.

Genomic DNA was isolated from whole blood using a QIAamp DNA Blood Mini Kit (Qiagen; Hilden, Germany) following standard procedures and quantified using a UV-visible spectrophotometer (NanoDrop 1000; Thermo Scientific, Waltham, MA, USA). Patient samples were genotyped using the TaqMan SNP genotyping assay (Applied Biosystems; Waltham, MA, USA) and the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) as described previously [23].

A total volume of 25 μL reaction mixture was used, containing 12.5 μL 2× Taq Master Mix (Takara; Shiga, Japan), 50 ng DNA, 3 pmol of each probe (Applied Biosystems) to perform polymerase chain reaction (PCR) amplification of LXR and ABCA1. The PCR conditions consisted of 40 cycles of 92°C for 15 seconds, and 60°C for 60 seconds after incubation at 95°C for 10 minutes. All primers used to detect SNPs were synthesized by Applied Biosystems. To confirm the accuracy of genotyping, randomly selected PCR products were verified by DNA sequencing performed by TsingKe Biological Technology (Beijing, China), and the results were confirmed by TaqMan genotyping. Sequences of primers used for PCR were rs7120118, 5′-TGTGCTGCCTGGAT GTATTG-3′ (forward) and 5′-CTCTGAGGGTC TGCT GATGC-3′ (reverse), and rs2230806, 5′-GTGTCCTGTCA TTGTGCCTTGT-3′ (forward) and 5′-CTCCCAGCCAG CCGTACTTT-3′ (reverse).

2.3. Statistical Analyses. As non-Gaussian distributed data, clinical data were presented as median and interquartile range and underwent chi-square testing to detect differences in patient clinical characteristics. Hardy-Weinberg equilibrium analysis of both SNPs and the genotype and allelic frequencies of SNPs were compared using the chi-square test. Association between each SNP and susceptibility to DKD were estimated by multivariate logistic regression analysis of three genetic models (additive, dominant, and recessive), correcting for age and sex. Next, multivariate logistic regression analysis was also used to evaluate the combined effect of both *LXR-α* rs7120118 and *ABCA1* rs2230806 polymorphism on DKD. An example to define these genetic models is the rs7120118 SNP, where T is the minor allele and C is the major allele. An additive genetic model was assumed, coding CC, TC, and TT as the number of copies of the minor allele (0, 1, or 2). In the dominant model, TT and TC were coded as 1, and CC was coded as 0. In the recessive model, TT was coded as 1, and TC and CC were coded as 0.

3. Results

3.1. Baseline Characteristics. In total, 646 participants were included in this study. The study consisted of two groups: DKD patients ($n = 300$; males 184, females 116) and DM patients ($n = 346$; males 199, females 145) (Table 1). All clinical characteristics including BMI, history of hypertension, blood pressure, Scr, TC, TG, and LDL-C were found to be elevated in DKD patients as compared with controls (Table 1).

3.2. Genotype and Allele Distributions of LXR-α rs7120118 and ABCA1 rs2230806 Polymorphisms. In terms of the rs7120118 and rs2230806 polymorphisms genotype and

Table 1: Demographics and clinical characteristics of DM patients with and without kidney diseases.

Variables	DM ($n = 346$)[a]	DKD ($n = 300$)[a]	P
Age, y	60.0 (53.0, 67.0)	62.5 (54.0, 71.0)	.003
Sex, male (%)	57.51 (199 (1)/346)	61.33 (184 (1)/300)	.336
BMI, kg/m^2	25.32 (23.08, 27.68)	25.80 (23.81, 28.18)	.021
Duration of diabetes, y	13.0 (10.0, 18.0)	15.0 (9.0, 20.0)	.149
History of hypertension (%)	51.16 (177/346)	78.0 (234/300)	<.001
SBP (mm Hg)	126.0 (120.0, 140.0)	140.0 (126.25, 150.0)	<.001
DBP (mm Hg)	80.0 (70.0, 80.25)	80.0 (74.0, 84.0)	.085
Scr (μmol/L)	62 (52.0, 72.15)	84.55 (64.93, 141.93)	<.001
TC (mmol/L)	4.16 (3.47, 4.88)	4.28 (3.50, 5.04)	.093
TG (mmol/L)	1.49 (1.03, 2.23)	1.70 (1.20, 2.58)	.011
LDL-C (mmol/L)	2.40 (1.93, 3.03)	2.42 (1.88, 2.96)	.898
HDL-C (mmol/L)	1.01 (0.83, 1.21)	0.96 (0.78, 1.17)	.427

$P < .05$ indicates statistical significance. [a]Data are shown as median (interquantile range) or %. Abbreviations: BMI: body mass index; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; SBP: systolic blood pressure; TC: total cholesterol; TG: triglyceride.

allelic frequencies in patients with DKD and controls, both groups were within Hardy-Weinberg equilibrium ($P > .05$), and their minor allele frequencies were over 5% (Table 2). Significant differences in the genotype and allele frequencies of the rs7120118 polymorphisms were detected between the DKD and DM groups (genotype, $P = .027$; allele, $P < .001$). In particular, the significant association between the minor allele (T) of rs7120118 and a decreased risk of DKD was identified in the allele ($P < .001$). The genotype frequencies of the rs2230806 were different between the two groups. No significant differences in allele frequencies of the rs2230806 were observed between the DKD and DM groups (genotype, $P = .046$; allele, $P = .259$).

3.3. Association of LXR-α rs7120118 and ABCA1 rs2230806 Polymorphisms with DKD. Three kinds of statistical models were applied to test the genotypic associations of *LXR-α* rs7120118 and *ABCA1* rs2230806 polymorphisms with DKD (Table 3). We hypothesized that the minor alleles of both SNPs were the risk factors as compared with the common alleles. Multivariate logistic regression analysis was performed (Table 3). When the rs7120118 CC genotype was used as the reference, a significantly decreased risk of DKD was associated with the TC+TT genotype (TC+TT versus CC: odds ratio (OR), 0.684; 95% CI 0.501-0.933; $P = .017$) in the dominant model. When the rs7120118 CC homozygote genotype was used as the reference, the TC and TT genotypes were associated with a decreased risk of DKD (TC versus CC: OR, 0.719; 95% CI 0.522-0.990; TT versus CC: OR, 0.460; 95% CI 0.224-0.946; trend $P = .043$) in the additive model. With the rs7120118 CC+TC genotype as reference, we found that the TT genotype was not associated with the risk of DKD (TT versus CC+TC: OR, 0.582; 95% CI 0.292-1.158; $P = .123$) in the recessive model. After adjusting for sex and age, the results were similar. Only the rs2230806 AA+AG genotype was significantly associated with increased risk of DKD with the Holm-Bonferroni correction ($P < .03$). There was also no statistically significant association between *ABCA1* rs2230806 and the risk of DKD under the other genetic models.

3.4. Combined Effect of LXR-α rs7120118 and ABCA1 rs2230806 Polymorphisms on DKD. The patients with the rs7120118 CC+rs2230806 AA genotype were used as reference. We found through multivariate logistic regression analysis that patients with rs7120118 CC+rs2230806 GG genotype showed an increased risk of DKD (OR, 2.531; 95% CI 1.262-5.078; $P = .009$; Table 4). No combined effect of other genotypes demonstrated an association with the risk of DKD. Results were similar after adjusting for age and sex (Table 4).

3.5. Genotype and Allele Distributions of ABCA1 rs2230806 Polymorphisms in Patients without Hypercholesterolemia. We summarize the rs2230806 polymorphisms genotype and allelic frequencies in patients with or without hypercholesterolemia. The genotype frequencies of rs2230806 were different between DKD and DM without hypercholesterolemia groups; however, no significant differences in rs2230806 allele frequencies were observed in the two groups (genotype, $P = .042$; allele, $P = .220$). No other differences were demonstrated in the genotype and allele frequencies of the rs2230806 polymorphisms between the DKD and DM patients with hypercholesterolemia (Table 5).

Three statistical models were used to test the genotypic associations of *ABCA1* rs2230806 polymorphisms with DKD with or without hypercholesterolemia (Table 5). For rs2230806, the minor allele is the risk allele. With the rs2230806 CC+TC genotype as reference, the TT genotype was not associated with the risk of DKD (TT versus CC+TC: OR, 0.497; 95% CI 0 .233-1.058; $P = .070$) in the recessive model. Adjusting for age and sex did not change the results. Only the TC+TT genotype was associated with a significantly increased risk of DKD without hypercholesterolemia ($P < .02$).

Multivariate logistic regression analysis revealed that when the rs2230806 AA+AG genotype was used as the reference, a significantly increased risk of DKD was associated

Table 2: Genotype and allele frequency of SNPs rs7120118 and rs2230806 between DM controls ($n = 346$) and DKD patients ($n = 300$).

	Genotype frequencies, *n* (%)				Allele frequencies, *n* (%)		
rs7120118	CC	CT	TT	*P*	C	T	*P*
DM	161 (46.5%)	160 (46.2%)	25 (7.2%)	.027*	482 (69.7%)	210 (30.3%)	.011*
DKD	168 (56.0%)	120 (40.0%)	12 (4.0%)		456 (76.0%)	144 (24.0%)	
rs2230806	AA	AG	GG	*P*	A	G	*P*
DM	86 (24.9%)	190 (54.9%)	70 (20.2%)	.046*	362 (52.3%)	330 (47.7%)	.259
DKD	78 (26.0%)	139 (46.3%)	83 (27.7%)		295 (49.2%)	305 (50.8%)	

*$P < .05$. Abbreviations: DM: diabetes mellitus; DKD: diabetic kidney disease.

Table 3: Genetic model analyses of the association between the SNPs and DKD with adjustment for age and gender.

	Genetic models	Genotype	DM	DKD	Without adjustment OR (95% CI)	*P*	With adjustment¶ OR (95% CI)	*P*
rs7120118	Additive	CC	161 (46.5%)	168 (56.0%)	1#		1#	
		TC	160 (46.2%)	120 (40.0%)	0.719 (0.522-0.990)	.043*	0.711 (0.514-0.984)	.040*
		TT	25 (7.2%)	12 (4.0%)				
	Dominant	CC	161 (46.5%)	168 (56.0%)	1#	.017*	1#	.015*
		TC+TT	185 (53.5%)	132 (44.0%)	0.684 (0.501-0.933)		0.677 (0.494-0.928)	
	Recessive	CC+TC	321 (92.7%)	288 (96.0%)	1#	.123	1#	.130
		TT	25 (7.2%)	12 (4.0%)	0.582 (0.292-1.158)		0.585 (0.293-1.171)	
rs2230806	Additive	AA	86 (24.9%)	78 (26.0%)	1#		1#	
		AG	190 (54.9%)	139 (46.3%)	0.807 (0.554-1.175)	.263	0.819 (0.560-1.197)	.302
		GG	70 (20.2%)	83 (27.7%)				
	Dominant	AA	86 (24.9%)	78 (26.0%)	1#	.739	1#	.842
		AG+ GG	260 (75.1%)	222 (74%)	0.941 (0.660-1.343)		0.964 (0.674-1.380)	
	Recessive	AA + AG	276 (79.8%)	217 (72.3%)	1#	.027*	1#	.018*
		GG	70 (20.2%)	83 (27.7%)	1.508 (1.048-2.171)		1.563 (1.081-2.260)	

*$P < .05$. ¶Adjustment for age and gender. #Reference category (odds ratio, 1.0). Abbreviations: CI: confidence interval; DKD: diabetic kidney disease; DM: diabetes mellitus; ORs: odds ratios.

Table 4: The combined effect of *LXR-α* rs7120118 and *ABCA1* rs2230806 polymorphisms on DKD.

Genotypes rs7120118	rs2230806	DM 346	DKD 300	Without adjustment OR (95% CI)	*P*	With adjustment¶ OR (95% CI)	*P*
CC	AA	46	45	1#	—	1#	—
CC	AG	97	81	0.854 (0.515-1.461)	.540	0.882 (0.529-1.469)	.629
CC	GG	18	42	2.385 (1.198-4.747)	.013*	2.531 (1.261-5.078)	.009*
CT+TT	AA	40	33	0.843 (0.455-1.564)	.589	0.853 (0.456-1.595)	.619
CT+TT	AG	93	58	0.638 (0.377-1.079)	.093	0.641 (0.377-1.092)	.102
CT+TT	GG	52	41	0.806 (0.451-1.440)	.466	0.845 (0.470-1.520)	.574

*$P < .05$. ¶Adjustment for age and gender. #Reference category (odds ratio, 1.0). Abbreviations: DM: diabetes mellitus; DKD: diabetic kidney disease.

with the GG genotype (AA+AG versus GG: odds ratio (OR), 1.568; 95% CI 1.070-2.296; $P = .021$) in the recessive model. With the rs2230806 AA genotype was used as the reference, the AG+GG genotype was not associated with the risk of DKD (AG+GG versus AA: OR, 0.966; 95% CI 0.666-1.401; $P = .854$) in the dominant model. Adjusting for age and sex did not change the results. Only the GG genotype showed association with a significantly higher risk of DKD without hypercholesterolemia ($P < .03$).

4. Discussion

The major novel finding of the present study is that *LXR-α* rs7120118 is significantly associated with DKD in Han Chinese patients and confirms the association of its minor allele (C) with decreasing DKD risk. More importantly, we found that the patients with *LXR-α* rs7120118 CC and *ABCA1* rs2230806 GG genotype showed an increased risk of DKD.

Table 5: Genetic model analyses of the association between SNP rs2230806 and DKD patients without hypercholesterolemia with adjustment for age and gender.

	Genetic models	Genotype	DM	DKD	P	Without adjustment OR (95% CI)	P	With adjustment[¶] OR (95% CI)	P
rs2230806	Additive	AA	82 (25.0%)	68 (25.7%)		1[#]		1[#]	
		AG	181 (55.2%)	123 (46.4%)	.042*	0.819 (0.552-1.216)	.043*	0.834 (0.560-1.243)	.031*
		GG	65 (19.8%)	74 (27.9%)					
	Dominant	AA	82 (25.0%)	69 (25.7%)	—	1[#]	.854	1[#]	.972
		AG+GG	248 (75.0%)	196 (74.3%)		0.966 (0.666-1.401)		0.993 (0.682-1.447)	
	Recessive	AA+AG	276 (70.2%)	217 (72.1%)	—	1[#]	.021*	1[#]	.013*
		GG	65 (19.8%)	74 (27.9%)		1.568 (1.070-2.296)		1.634 (1.110-2.407)	

*$P < .05$. [¶]Adjustment for age and gender. [#]Reference category (odds ratio, 1.0). Abbreviations: CI: confidence interval; DKD: diabetic kidney disease; DM: diabetes mellitus; ORs: odds ratios.

Dyslipidemia has been identified to promote the progression of DKD [24]. Our previous research [13] and an increasing number of studies [25, 26] have placed further importance on cholesterol accumulation in the DKD kidney and are considered a risk factor of lipid metabolism disorder, which contributes to renal injury. As an important regulator of lipid metabolism, LXRs are the key mediator of lipid homeostasis, including maintaining lipid balance [27], and preventing or slowing atherosclerosis [28]. They are also important in regulating immunity [29] and exerting anti-inflammatory properties [30]. In our previous study, we found renal lipid deposition and kidney injury was aggravated by downregulation of LXR-α. However, the effectiveness of LXR agonists is limited by serious side effects, such as liver steatosis, hyperlipidemia, and impairment of neutrophil functions [31]. Interestingly, Akiyama et al. found that functional genes at homologous loci identified using human lipid GWASs responded to an animal model with high-fat and high-cholesterol diet intervention [32]. They observed a significant association of the *LXR-α* rs7120118 with serum lipid levels. Grzegorzewska et al. found that patients carrying the allele T at rs7120118 were lower in the hemodialysis patients with atherogenic dyslipidemia than in those without atherogenic dyslipidemia [10]. Similarly, our study showed that genotypic and allelic frequencies of rs7120118 were different between DM and DKD patients. And, patients with DKD had lower frequencies of rs7120118 T allele than the controls. In particular, the genotype and allele frequencies of rs7120118 SNP were also associated with the risk of DKD for different genetic models. In DKD patients, the rs7120118 TC+TT genotype was associated with a low risk of DKD (TC+TT versus CC), and the rs7120118 TC and TT genotypes were associated with a decreased risk of DKD (TC versus CC; TT versus CC) compared with DM patients. Therefore, dyslipidemia might be involved in the risk of *LXR-α* rs7120118 on DKD. However, Wu et al. demonstrated no significant association between rs7120118 genotype and risk of coronary heart disease in the Chinese Han population they studied [11].

Since ABCA1 promotes solubilization of lipids and their release, increasing ABCA1 should mediate efflux of free cholesterol from cells which is the early step in reverse cholesterol transport [33]. The molecular defect in the *ABCA1* gene results in Tangier disease, which is characterized by HDL deficiency, proteinuria, and premature atherosclerosis [34]. Additionally, some studies report that the genetic variants of ABCA1 are significantly associated with an individual's risk of developing coronary artery disease [35, 36]. In patients with DKD, a decrease in ABCA1 leads to an increase in cholesterol accumulation in renal tissues [34]. Similar changes in renal tissues were reported in diabetic apolipoprotein E knockout (apoE$^{-/-}$) and db/db mice [30, 34, 37]. Additionally, because of the anti-inflammatory properties of ABCA1, its dysfunction tends to cause chronic low-grade inflammation in patients [38, 39]. Therefore, improving function or upregulating ABCA1 expression is sufficient to extenuate diabetic kidney injury. Studies have demonstrated that the *ABCA1* rs2230806 polymorphism is significantly associated with patients with severe dyslipidemia [40], such as coronary heart disease [41] and obesity [42], as well as with DM [19]. Meta-analysis by Jung et al. found that *ABCA1* rs2230806 polymorphism was significantly associated with DM in Asians [19]. Unfortunately, our results indicated that rs2230806 was not independently associated with the analyzed genotypes between the DM and DKD in a Chinese Han population.

As metabolite-sensing receptors, ABCA1 may have a close correlation with serum lipid levels. As such, we conducted subgroup analysis on hypercholesterolemia to test the genotypic associations of rs2230806 polymorphisms with DKD. Of further interest, the genotype frequencies of the rs2230806 were different between DM and DKD without hypercholesterolemia groups. The rs2230806 AG and GG genotypes were associated with a higher risk of DKD (AG versus AA; GG versus AA) compared with DM patients without hypercholesterolemia.

In conclusion, the current study suggests that *LXR-α* rs7120118 is significantly associated with the risk of DKD and confirms the association of its minor allele (T) with decreasing DKD risk. *ABCA1* rs2230806 is significantly associated with the risk of DKD and confirms its minor allele G as a higher risk factor for DKD without hypercholesterolemia. The association of LXR-α-ABCA1 is highly interesting in DKD. This genetic tool could identify high-risk DM patients who need closer monitoring to prevent or slow progression to DKD. Further studies are required, however, to investigate the biological mechanisms underlying this relationship.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

All the authors declared no competing interests.

Authors' Contributions

Peng Liu and Liang Ma contributed equally to this work.

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