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Characterisation of key biomarkers in diabetic ulcers via systems bioinformatics

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Abstract

Diabetic ulcers (DUs) are characterised by a high incidence and disability rate. However, its pathogenesis remains elusive. Thus, a deep understanding of the underlying mechanisms for the pathogenesis of DUs has vital implications. The weighted gene co-expression network analysis was performed on the main data from the Gene Expression Omnibus database. Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were adopted to analyse the potential biological function of the most relevant module. Furthermore, we utilised CytoHubba and protein–protein interaction network to identify the hub genes. Finally, the hub genes were validated by animal experiments in diabetic ulcer mice models. The expression of genes from the turquoise module was found to be strongly related to DUs. GO terms, KEGG analysis showed that biological functions are closely related to immune response. The hub genes included IFI35, IFIT2, MX2, OASL, RSAD2, and XAF1, which were higher in wounds of DUs mice than that in normal lesions. Additionally, we also demonstrated that the expression of hub genes was correlated with the immune response using immune checkpoint,

Abbreviations: AGEs, advanced glycation end products; CNS, central nervous system; DAMPs, damage-associated molecular patterns; DEGs, differentially expressed genes; DUs, diabetic ulcers; GEO, Gene Expression Omnibus; GO, Gene Ontology; HUVEC, human umbilical vein endothelial cells; IAP, inhibitor of apoptosis protein; IFI35, interferon induced protein 35; IFIT2, Interferon Induced Protein With Tetratricopeptide Repeats 2; ISG, interferon stimulated genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; MX2, MX Dynamin Like GTPase 2; NO, nitric oxide; OASL, 2′-5′-Oligoadenylate Synthetase Like; PAD4, peptidyl arginine-deiminase 4; PPI, protein–protein interaction; qPCR, quantitative real-time polymerase chain reaction; RSAD2, Radical S-Adenosyl Methionine Domain Containing 2; SPF, specific pathogen-free animal; ssGSEA, single-sample gene set enrichment analysis; TCR, T-cell receptor; VEGF, vascular endothelial growth factor; WCGNA, weighted gene co-expression network analysis; XAF1, XIAP Associated Factor 1.

Zhan Zhang, Ying Zhang, and Dan Yang contributed equally to this study.

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immune cell infiltration, and immune scores. These data suggests that IFI35, IFIT2, MX2, OASL, RSAD2, and XAF1 are crucial for DUs.

KEYWORDS

bioinformatics, biomarkers, diabetes complications, ulcer

Key Messages

- weighted gene co-expression network analysis was performed on the main data from the Gene Expression Omnibus database to identify the most relevant module of diabetic ulcers
- Gene Ontology terms, Kyoto Encyclopedia of Genes and Genomes analysis demonstrated the most relevant module is strongly associated with immune response
- construct the protein–protein interaction network and identify the hub genes including IFI35, IFIT2, MX2, OASL, RSAD2, and XAF1
- the hub genes had higher expressions in wounds of diabetic ulcers mice

1 | INTRODUCTION

Over the past 40 years, the number of patients with diabetes has rapidly increased worldwide.[1] Chronic hyperglycemia leads to long-term complications of diabetes, including heart diseases, stroke, retinopathy, nephropathy, and diabetic ulcers (DUs).[2-5] 19%–34% of diabetes patients will develop DUs in their lifetime.[6] In particular, 21.7% of diabetes patients had an amputation or died, with a higher proportion among people with a pre-existing condition of DUs compared to those who have no pre-existing condition of DUs.[7]

Compared with healthy people, the wound healing of diabetes patients is delayed because the healing mechanism is inhibited by several factors, such as neuropathy, peripheral angiopathy, and impaired immunity.[8] Wound healing is a complex biological process involving a large number of cell types, cytokines, and growth factors.[8] Recently researchers find that DU is characterised by chronic inflammation and impaired angiogenesis.[9,10] The inflammatory cytokine IL-1β and TNF-α are increased in chronic human and mice wounds.[11] The acute inflammatory reaction is the first process of wound healing. An array of cytokines and chemokines attract inflammatory cells including neutrophils and macrophages to the site of injury and inflammation, which recruits growth factors.[12] In the early stages of wound healing, neutrophils can defend against invading pathogens. However, prolonged inflammation, neutrophil, and neutrophil-derived proteases are often associated with chronic non-healing or slow-healing wounds.[13-15] It has been suggested that the peptidylarginine-deiminase 4 (PAD4) protein, which was enhanced in neutrophils of diabetic patients, is associated with a reduction in inflammation and tissue damage.[15] And studies have revealed that hyperglycemia and excessive advanced glycation end products (AGEs) formation prevent the clearing of neutrophils by macrophages, thereby continuing a pro-inflammatory state.[16,17]

Angiogenesis is important for granulation tissue formation during wound healing and can also help other cells' proliferation and migration. However, because of the low expression of vascular endothelial growth factor (VEGF) and hypoxia, this process is inhibited in DU.[18] Numerous studies have shown that hyperglycemia leads to an impairment of nitric oxide (NO) production and activity in diabetic patients, leading to the development of endothelial dysfunction and impaired angiogenesis.[19] And it has been reported that in wound healing, platelet dysfunction induced by diabetes may prevent normal healing processes.[20] Platelets are central to haemostasis, helping to form a hemostatic plug or thrombus without occluding the vessel. During wound healing, the thrombus has to be degraded so that the re-epithelialization can start. Yet, insulin resistance and abnormal glucose metabolism could further increase platelet aggregation.[21] In addition, the inhibitory effect of NO released from vascular endothelial cells to platelet aggregation is decreased, which reduces angiogenesis.[22]

The major treatments for DUs include clearing necrotic tissue, antibiotics, wet dressing therapy, negative pressure therapy, and hyperbaric oxygen.[23] However, some patients have a high rate of recurrence with a poor

TABLE 1 The primers used for quantitative real-time polymerase chain reaction (qPCR)

Name	Primer sequence	Size (bp)
m IFI35-QPCR-F	AACCACAGGGTGCTCGTTAG	202
m IFI35-QPCR-R	TGAACTGGCCAATCTGGCAT	
m IFIT2-QPCR-F	GGGAGTCCCCCAAAGGACTA	225
m IFIT2-QPCR-R	GGCCAGAACTTGCTTTTCGG	
m MX2-QPCR-F	CTGACCGCAGAGCTCATCTT	253
m MX2-QPCR-R	ACTCTTTTCGGAGCCTGGTG	
m OASL-QPCR-F	TTTCTCCAAGGAGGGAGGGG	278
m OASL-QPCR-R	TCTCGATTCTCTCCCAGGCA	
m RSAD2-QPCR-F	AGCAGGTGTGTGCCTATCAC	229
m RSAD2-QPCR-R	GCTGAGTGCTGTTCCCATCT	
m XAF1-QPCR-F	GAAGCTTGACCATGGAGGCT	200
m XAF1-QPCR-R	GTGCTGTTGGCTTTCCTTGG	

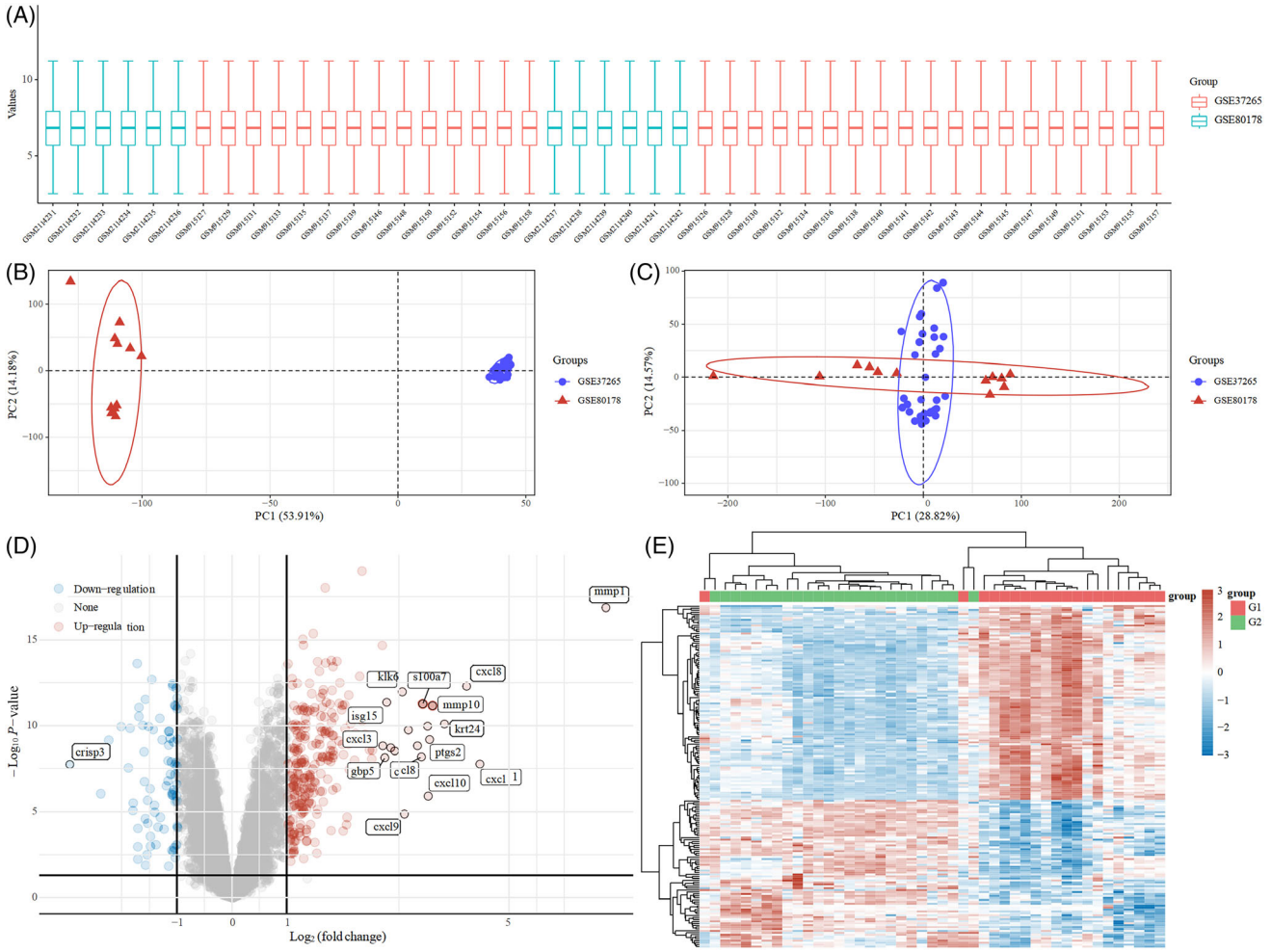

FIGURE 1 Bioinformatic analysis of the differentially expressed genes (DEGs) between healthy volunteers and diabetic ulcer patients. In (A) Box plot after data standardisation, different colours represent different data sets. (B) Principal component analysis (PCA) results before batch removal for multiple data sets. Different colours represent different data sets. (C) PCA results after batch removal, showing the intersection of these two data sets. Different colours represent different data sets. (D) Volcano plots of DEGs were constructed to adjust to fold-change and *P* value. The red points represent the over-expressed mRNAs and the blue points indicate the down-expressed mRNAs. (E) Heatmap of the top 100 DEGs. Different colours represent the different expression trends

prognosis. Desperately needed work is to gain new insight into the pathogenesis of DUs.

Systems Bioinformatics is a relatively emerging field that integrates information including systems biology and classical bioinformatics.[20] Bioinformatics manages and analyses biological data through computational technologies. In order to study more associations between multi-omics (genomics, transcriptomics, proteomics, and metabolomics) data and diseases, these data sets must be integrated and analysed as a holistic system by bioinformatics.[20] With the gene mapping techniques and bioinformatics analysis developing further and refining, we can identify the variation of gene expression caused by DUs through bioinformatics analysis, and thereby find the core biomarkers and molecular mechanisms of DUs. Although a previous bioinformatics analysis that included DUs was performed.[24] However, owing to the different objectives they reveal the biomarkers between acute trauma and chronic wounds, and Rong et al. only explored the module which was highly related to the immune score. Thus, the impact of DUs compared with normal has not been fully assessed.

Therefore, the purpose of comprehensive bioinformatics analysis focusing on DUs is to clarify the core biomarkers and modules of DUs and to know about immune correlates. In addition, experimental validation on DUs was adopted to give significant basic research evidence. The current study will provide new insights into the underlying mechanisms for the pathogenesis of DUs.

2 | METHODS

2.1 | Data collection

The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), an international public repository, can archive and freely distribute high-throughput gene expression and other functional genomics datasets. Two databases including GSE37265 (Affymetrix Human Genome

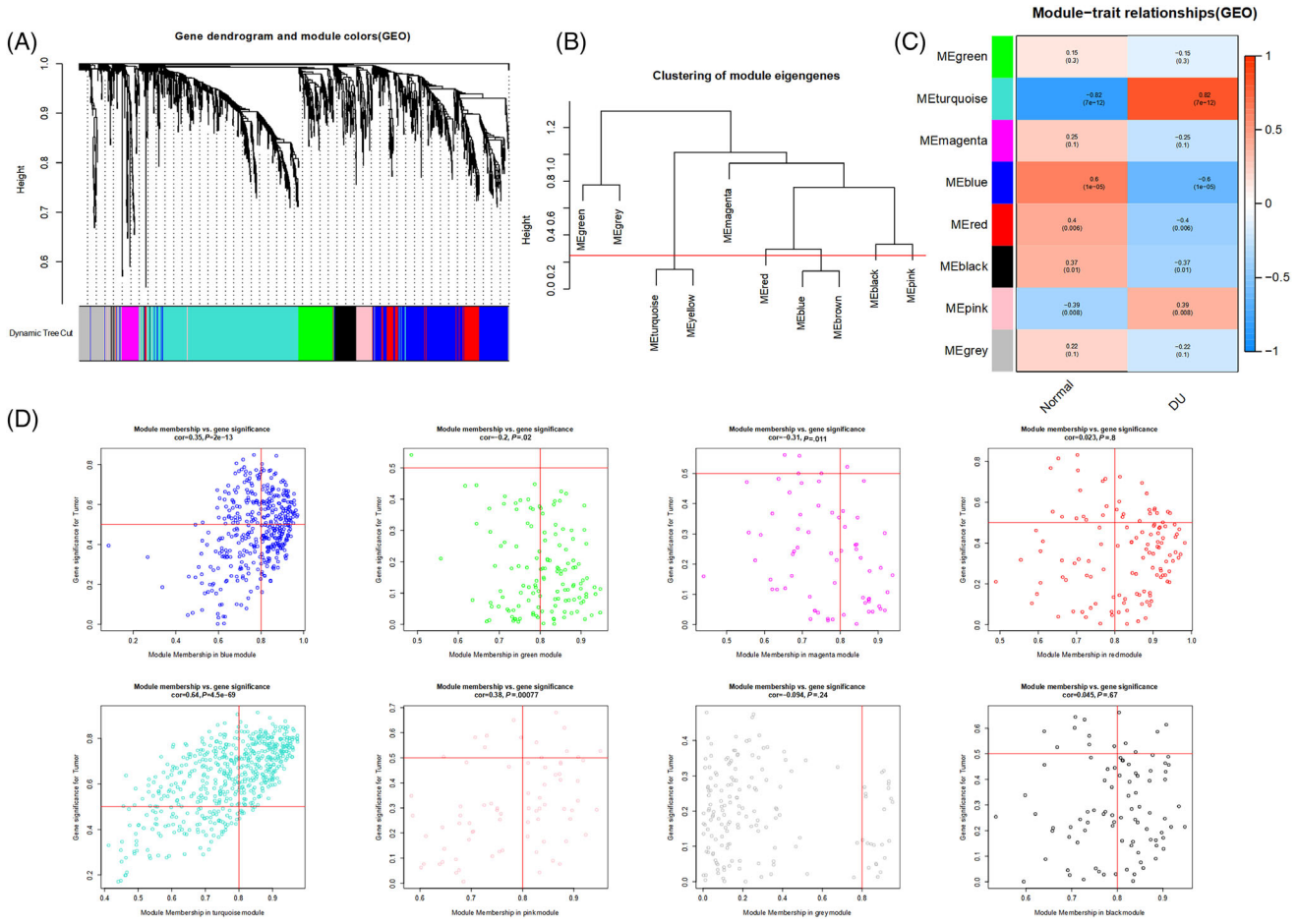

FIGURE 2 Weighted gene co-expression analysis. (A) Gene cluster dendrogram clustered by weighted gene co-expression network in different colours. (B) 10 co-expression modules. (C) The heatmap of module-trait relationships between healthy volunteers and diabetic ulcers. Number in and outside the bracket represents *P*-value and Pearson coefficient, respectively. (D) Correlation analysis between module membership and gene significance in the turquoise modules

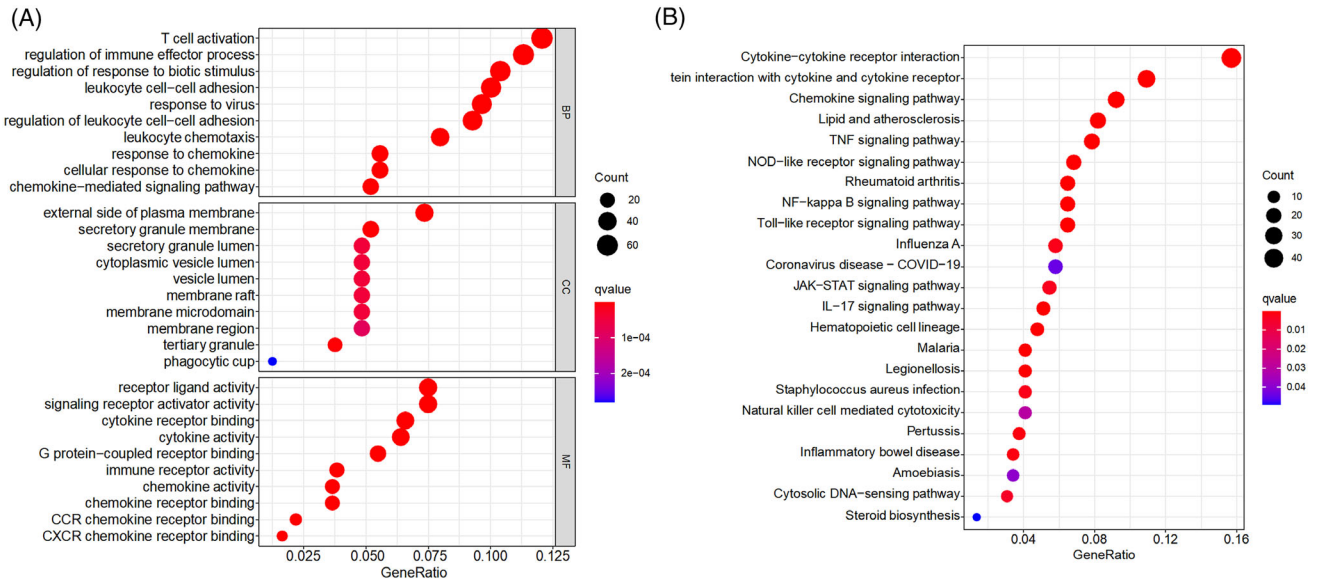

FIGURE 3 Functional enrichment of ulcer-related genes. (A) Gene Ontology analysis of differentially expressed genes involving the biological processes, cellular components, and molecular functions. (B) Kyoto Encyclopedia of Genes and Genomes showing the signalling pathway associated with diabetic ulcers

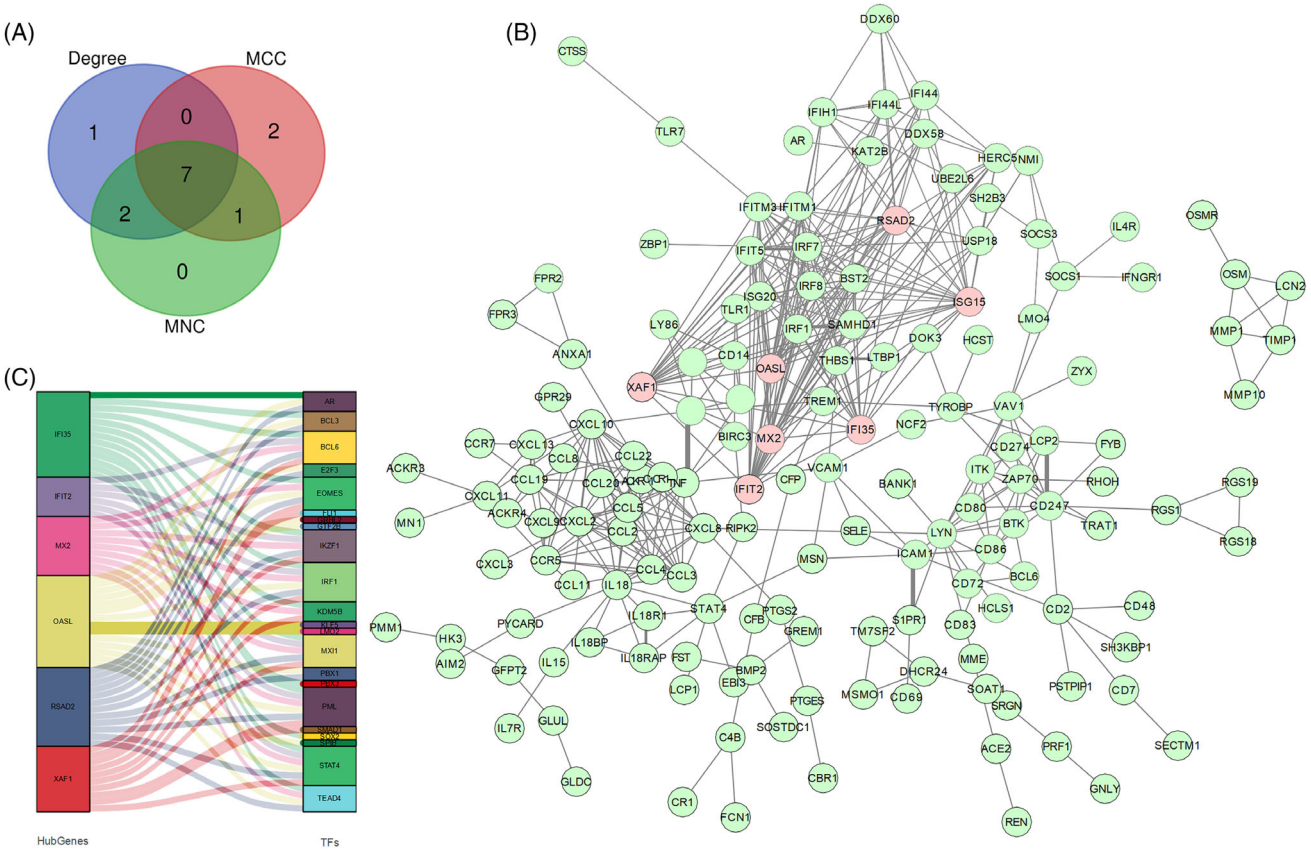

FIGURE 4 PPI network construction and hub gene identification. (A) Venn Diagram of the hub genes identified using three graph-theoretic algorithms including Degree, MCC, and MNC. (B) Protein–protein interaction network of the hub genes. (C) Sankey diagram for the correlation analysis of transcription factors and hub gene in ulcer. Each rectangle represents a gene, and the connection degree of each gene is displayed based on the size of the rectangle

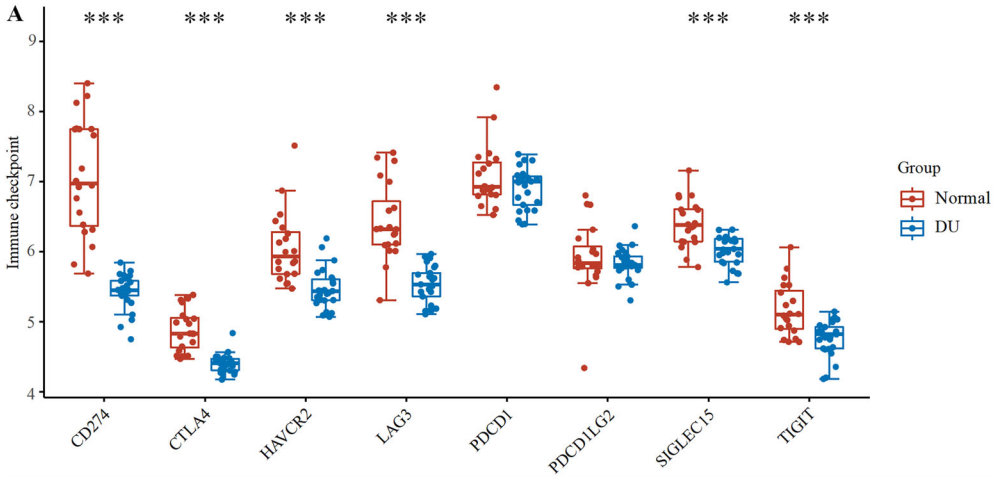

FIGURE 5 The expression distribution of immune checkpoints related mRNAs in case and control groups, where the horizontal axis represents different mRNA and the vertical axis represents the mRNA expression distribution. Different colours represent different groups, and the upper left corner represents the significance *P*-value test method. ***$P < .001$

U133 Plus 2.0 Array, USA, 25 March 2019) and GSE80178 (Affymetrix Human Gene 2.0 ST Array, USA, 15 March 2019) were obtained from the GEO database for the expression of mRNA in DUs patients, and the download data format is MINIML. Inclusion criteria: all subjects of the included studies were human; samples not based on cell lines; sample type should be skin tissue; datasets with complete data for analysis; ethical approval was obtained; DUs or normal samples should be included; the control group without neither systemic nor autoimmune diseases nor relevant family history.

2.2 | Data processing

Screening for differentially expressed mRNA was performed using the Limma package (version: 3.40.2) of R software, and significance was indicated by |Log(fold change)| >1 and a corresponding *P*-value ≤.05. Further, a principal component analysis (PCA) was used to cluster trends with the R software.

2.3 | Weighted gene co-expression network

The weighted gene co-expression network (WGCNA) package in R was used to detect co-expressed gene modules, confirm the hub genes, and the association between the gene network and the disease phenotype. This approach is a particularly powerful means to indicate co-expression among disease-related genes, which has been reported previously.[25,26] The samples were clustered when the soft-threshold power was set as 4, height was set as 0.25, and the minimum module size was set as 30. Then extract the gene clusters' names and show them as a heatmap. The scatter plots showed the correlations between the genes and DUs.

TABLE 2 The differences of immune checkpoints in normal tissue and diabetic ulcers (DUs)

Immune checkpoint	Correlation with DUs	*P*-value
CD274	Negative	<.001
CTLA4	Negative	<.001
HAVCR2	Negative	<.001
LAG3	Negative	<.001
PDCD1	Not significant	>.1
PDCD1LG2	Not significant	>.1
SIGLEC15	Negative	<.001
TIGIT	Negative	<.001

2.4 | Functional enrichment analysis

To better understand the function of potential targets, the key module was analysed using functional enrichment analysis. Gene Ontology (GO) analysis is a major bioinformatics tool for annotating genes and gene functions. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a knowledge database for systematic analysis of gene function, linking genomic information to higher-order functional information. The GO and KEGG analysis were performed using the R package "cluster Profiler."

2.5 | Network construction

We performed three algorithms (degree, MCC, and MNC) in cytohubba, and got the Venn diagram by overlapping the three results. Protein–protein interaction (PPI) showed the overlapping hub genes. Sankey diagram shows the relationship between hub genes and transcription factors.

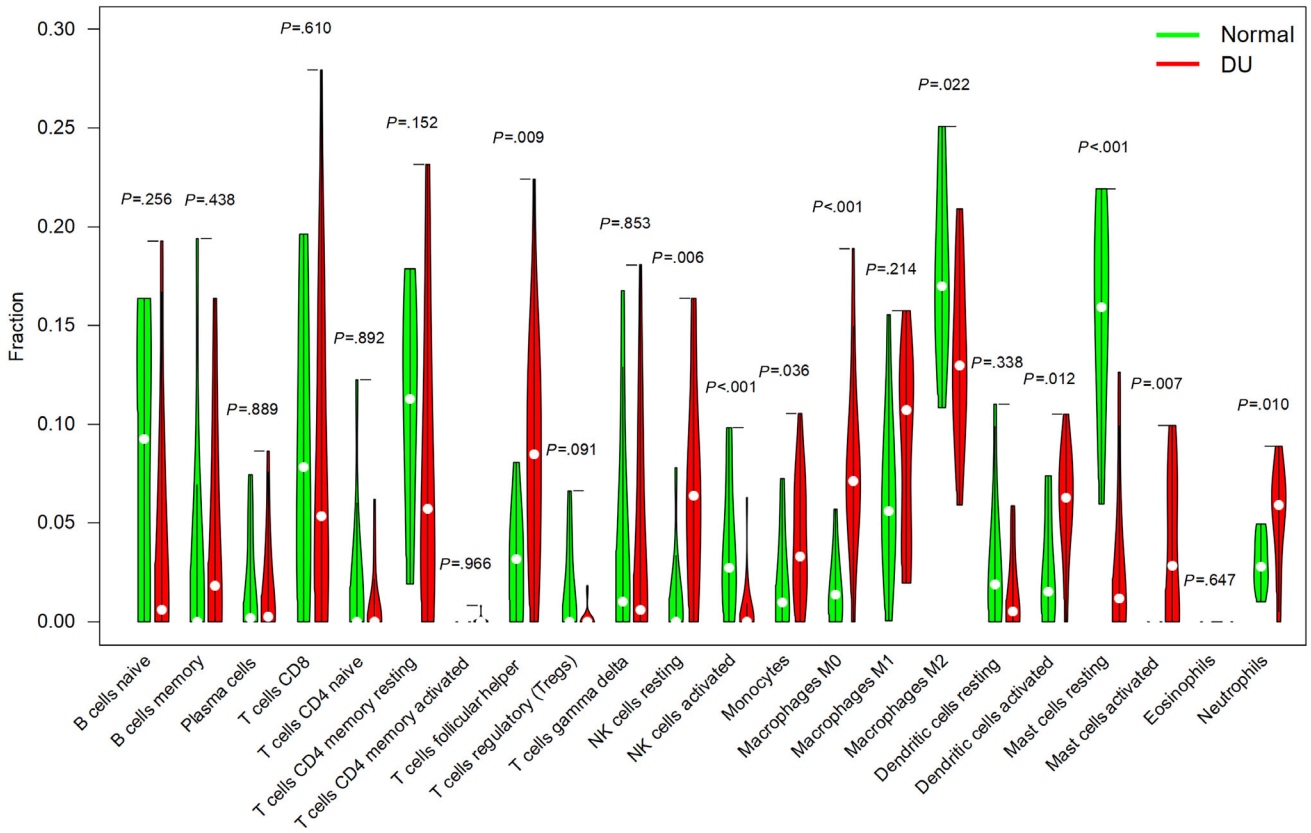

FIGURE 6 Violin plot of the difference of proportion in 22 immune cells between diabetic ulcers and normal tissue by single-sample gene set enrichment analysis analysis

2.6 | Correlation analysis of gene expression and immune function

To assess the level of immune cell infiltration and immune function in a single sample, a single-sample gene set enrichment analysis (ssGSEA) is performed according to the expression levels of immune cell-specific markers. The data of DU patients and healthy volunteers were imported for the ssGSEA analysis.

2.7 | Validation experiments

2.7.1 | Animals

Twenty female C57BL/6 (8 weeks old) obtained from Shanghai Slac Laboratory Animal Co., Ltd. (scxk Shanghai 2017-0005) were bred in the Laboratory Animal Center of Shanghai University of TCM. They had a high-fat diet (Shanghai Pu Lu Tong Biological Technology Co., Ltd) or standard diet and ad libitum access to water under controlled temperature (23 ± 2°C), in specific pathogen-free animal (SPF) grade cages. The mice were equally divided into the normal group and disease group.

2.7.2 | Diabetic ulcer mice model

A diabetic ulcer mice model was established in the current study. The model group mice and healthy group mice were injected intraperitoneally with 2% streptozotocin dissolved in a mix of citrate buffer. The blood glucose of the disease group was kept over 16.7 mmol/L. After shaving, full-thickness excisional skin wounds were created with a sterile biopsy punch in the back of mice. The mice were executed and skin tissue was taken on day 9 after punching. And all tissue was immediately put into liquid nitrogen and stored at −80°C.

2.7.3 | Quantitative real-time polymerase chain reaction

The mRNA expressions of hub genes were detected with quantitative real-time polymerase chain reaction (qPCR). Firstly, we used TRIzol reagent to extract total RNA. Secondly, the concentration of total RNA was determined by an ultraviolet spectrophotometer. Then, we reverse-transcribed 20 μL of total RNA to a cDNA probe using Reverse Transcription System First Strand cDNA Synthesis

Kit. PCR primers were listed in Table 1. And cDNA was used for qPCR analysis. After the reaction, the collected fluorescent quantitative data were analysed.

2.7.4 | Statistical analysis

All the values were expressed as means ± the SD. The one-way analysis of variance (ANOVA) and student *t*-test were applied to compare the differences between the groups. *P*-value <.05 was considered a significant statistical difference. All calculations were performed using SPSS 24.0 statistical software.

3 | RESULTS

3.1 | Identification of differentially expressed genes related to DUs patients

We retrieved and obtained the relevant data sets of DUs from GSE37265 and GSE80178 (Figure 1A). PCA was performed to show the relationship among the different data sets. As is shown in Figure 1B, before removing the batch the two data sets were separated without any intersection. The first principal component (PC1) accounted for 53.91% of the total variance, and the second principal component (PC2) accounted for 14.18%. However, the two sets intersected together after batch removal, and this means the two data sets can be analysed as a batch of data (Figure 1C). PC1 accounted for 28.82%, and PC2 accounted for 14.57% of the total variance. Figure 1D showed the result of the differential analysis, 8350 genes in total. The top 50 down−regulation genes and up-regulation genes were shown on the heatmap (Figure 1E).

3.2 | WGCNA and key module identification

As is shown in the hierarchical clustering tree of Figure 2A,B, WGCNA was used to cluster the similar modules, and we obtained 10 co-expression modules which were represented with different colours. The heatmap of module-trait relationships showed that the turquoise module has the greatest difference in the correlations between healthy volunteers and DUs (Figure 2C). In the turquoise module, the scatter plots in Figure 2D illustrated that module membership and gene significance have a strong correlation (cor = 0.64, $P = 4.5e{-}69$).

TABLE 3 The different immune cell occupancies in normal tissue and diabetic ulcers (DUs)

Immune fraction	Correlation with DUs	*P*-value
Naive B cell	Not significant	>.1
Memory B cell	Not significant	>.1
Plasma cells	Not significant	>.1
CD8 T cell	Not significant	>.1
CD4 naive T cell	Not significant	>.1
CD4 memory resting T cell	Not significant	>.1
CD4 memory activated T cell	Not significant	>.1
T cells follicular helper	Positive	<.05
T cells regulatory helper	Negative	<.1
γδ T cell	Not significant	>.1
Resting NK cells	Positive	<.05
Activated NK cells	Negative	<.001
Monocytes	Positive	<.05
Macrophages M0	Positive	<.001
Macrophages M1	Not significant	>.1
Macrophages M2	Negative	<.05
Resting dendritic cells	Not significant	>.1
Activated dendritic cells	Positive	<.05
Resting mast cells	Negative	<.001
Activated mast cells	Positive	<.05
Eosinophils	Not significant	>.1
Neutrophils	Positive	<.05

3.3 | Functional enrichment analysis of the turquoise module

To further explore the functions of hub genes and relevant pathways from the turquoise module, GO and KEGG enrichment was performed. In GO analysis, we mainly enriched T cell activation and regulation of immune effector process in the biological process, external side of the plasma membrane, and secretory granule membrane in terms of cell component, receptor-ligand activity, and signalling receptor activator activity in terms of molecular function (Figure 3A). KEGG enrichment analysis showed that the main pathways include cytokine−cytokine receptor interaction, protein interaction with cytokine and cytokine receptor, Chemokine signalling pathway, Lipid and atherosclerosis, and TNF signalling pathway (Figure 3B).

3.4 | PPI network construction and hub gene validation

Seven hub genes were identified from the PPI network and three different graph-theoretic algorithms were available in Cytohubba, including Degree, MCC, and MNC. The hub genes include IFI35, IFIT2, MX2, OASL, RSAD2, XAF1, and ISG15 (Figure 4A,B). Moreover, a Sankey diagram further demonstrated the correlation analysis of transcription factors and hub genes in DUs (Figure 4C).

3.5 | Immune checkpoints, immune cell infiltration, and immune function score in DUs

Figure 5 reveals the differences in several immune checkpoints between normal tissue and DUs. Compared with normal tissue, CD274, CTLA4, HAVCR2, LAG3, SIGLEC15, and TIGIT were decreased significantly in DUs, but PDCD1 and PDCD1LG2 were not significant (Table 2). To detect the difference in proportion in 22 immune cells between DUs and normal tissue, we performed the ssGSEA analysis (Figure 6). The violin plots affirmed that 10 types, including T cells follicular helper, resting NK cells, Monocytes, Macrophages M0, activated Dendritic cells, activated Mast cells, and Neutrophils, markedly elevated in DUs ($P < .05$). In contrast, activated NK cells, M2, and resting Mast cells were negatively correlated with DUs (Table 3). Then to investigate the difference in immune function status and performance status score between normal tissue and DUs, we compared the immune function score (Figure 7). And we found that the immune function scores of aDCs, APC co-inhibition, B cells, Check-point, CCR, Cytolytic activity, Inflammation−promoting, DCs, Macrophages, Neutrophils, Parainflammation, pDCs, T cell coinhibition, T cell costimulation, T helper cells, Th1 cells, Tfh, Treg, TIL, and Type I IFN Response were significantly higher in DUs than in normal tissue. However, the iDCs were lower in DUs. And there were no remarkable differences in APC costimulation, Mast cells, Th2 cells, Type II IFN Response (Table 4).

3.6 | Verification of differentially expressed genes in vivo

To confirm the role of hub genes, we performed the PCR to compare their mRNA expression between DUs

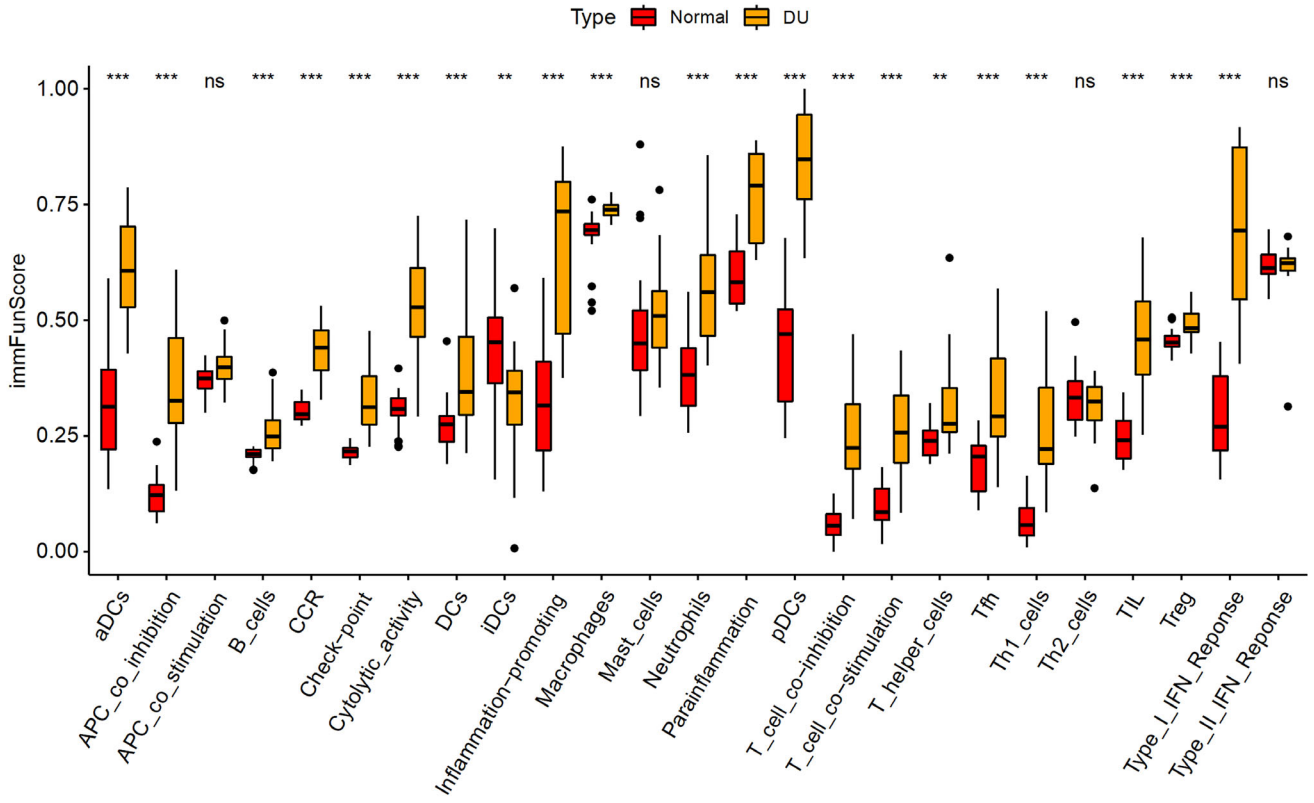

FIGURE 7 Difference analysis of immune function status and performance status score between ulcer patients and normal subjects in the Gene Expression Omnibus data set

TABLE 4 The differences of immune scores in normal tissue and diabetic ulcers (DUs)

Imunne score	Correlation with DUs	*P*-value
aDCs	Positive	<.001
APC co-inhibition	Positive	<.001
APC co-stimulation	Not significant	>.1
B cells	Positive	<.001
CCR	Positive	<.001
Check-point	Positive	<.001
Cytolytic activity	Positive	<.001
DCs	Positive	<.001
iDCs	Negative	<.05
Inflammation−promoting	Positive	<.001
Macrophages	Positive	<.001
Mast cells	Not significant	>.1
Neutrophils	Positive	<.001
Parainflammation	Positive	<.001
pDCs	Positive	<.001
T cell coinhibition	Positive	<.001
T cell costimulation	Positive	<.001
T helper cells	Positive	<.05
Tfh	Positive	<.001
Th1 cells	Positive	<.001
Th2 cells	Not significant	>.1
TIL	Positive	<.001
Treg	Positive	<.001
Type I IFN response	Positive	<.001
Type II IFN response	Not significant	>.1

mice and healthy mice. As is shown in Figure 8, IFI35, IFIT2, MX2, OASL, RSAD2, and XAF1 have higher expression in DUs ($P < .05$), compared with the healthy group.

4 | DISCUSSION

The incidence of DUs is rising every year in countries around the world.[27] DUs usually lead to limb loss or disability, bringing about tremendous pressure on the mind and economy for families and society.[27] The abnormal expression of various chemokines and growth factors in the skin of DUs results in slow healing of wounds.[28] In patients with type 2 diabetes, M1 macrophage (pro-inflammatory cells) is hard to transfer to M2 macrophage (anti-inflammatory cells), which causes chronic inflammation.[29] Pro-inflammatory cytokines including IL-1, IL-6, and TNF-α also increase remarkably.[30] Besides, wound non-healing is directly related to abnormal angiogenesis, and the deficiency of VEGF is an important factor.[31] However, the present therapeutic effects might not be satisfactory. So, finding the novel biomarkers and molecular mechanisms of DUs is a better way to find good therapeutics.

In this study, we performed the WGCNA to identify the key genes from 8350 genes obtained from two databases. PCA was performed to identify the two databases that can be analysed together. We got 10 modules. Correlation between genes in modules and clinical features may contribute to the understanding of the pathogenesis of DUs. The turquoise module was the most correlated co-expression module for DUs. The GO and KEGG analysis indicated the potential functions of the turquoise module were intensively enriched for immune response and inflammatory response such as T cell activation, regulation of immune effector process, cytokine-cytokine receptor interaction, and chemokine signalling pathway.

The combination of WGCNA, cytoHubba, and PPI network identified IFI35, IFIT2, OASL, RSAD2, MX2, and XAF1 as the hub genes. Interestingly, IFI35, IFIT2, OASL, RSAD2, and MX2 all belong to interferon-stimulated genes (ISG). ISG plays a crucial role in the cellular antiviral response and innate immune signalling. It was also strongly associated with inflammatory diseases.[32] They were verified to be significant genes by WGCNA, cytoHubba PPI network, and PCR. IFI35 (Interferon Induced Protein 35, IFP35) was not only activated multiple antiviral pathways in promoting antiviral activities, which has been widely reported in SARS-CoV-2 virus, vesicular stomatitis virus, and H5N1 virus,[33-35] but involved in chronic inflammatory diseases of the skin, kidney, and central nervous system (CNS).[32] IFI35 is considered as the pro-inflammatory factor and damage-associated molecular patterns (DAMPs) in vivo, driving the activation of the natural immune.[36] Moreover, IFI35 was up-regulated by TNF-α and IL-1β in endothelial cells.[37] And IFI35 suppressed the proliferation and migration of human umbilical vein endothelial cells (HUVEC), which was detrimental to wound healing.[38] Interferon Induced Protein With Tetratricopeptide Repeats 2 (IFIT2) is a Protein Coding gene. Although IFIT2 exhibited antiviral activity, increasing neurotropic coronavirus replication dramatically in the absence of IFIT2,[39] it has been reported that IFIT2 is instead repurposed by influenza virus to promote viral gene expression.[40] In addition, IFIT2 functions as tumour suppressor in multiple tumours.[41,42] It is reported that LPS increased IFIT2 in macrophages, and increased more when M1 macrophage polarisation.[43] Meanwhile, IFIT2 helped the LPS induce the expression of IL-6 and TNF-α.[44,45] OASL

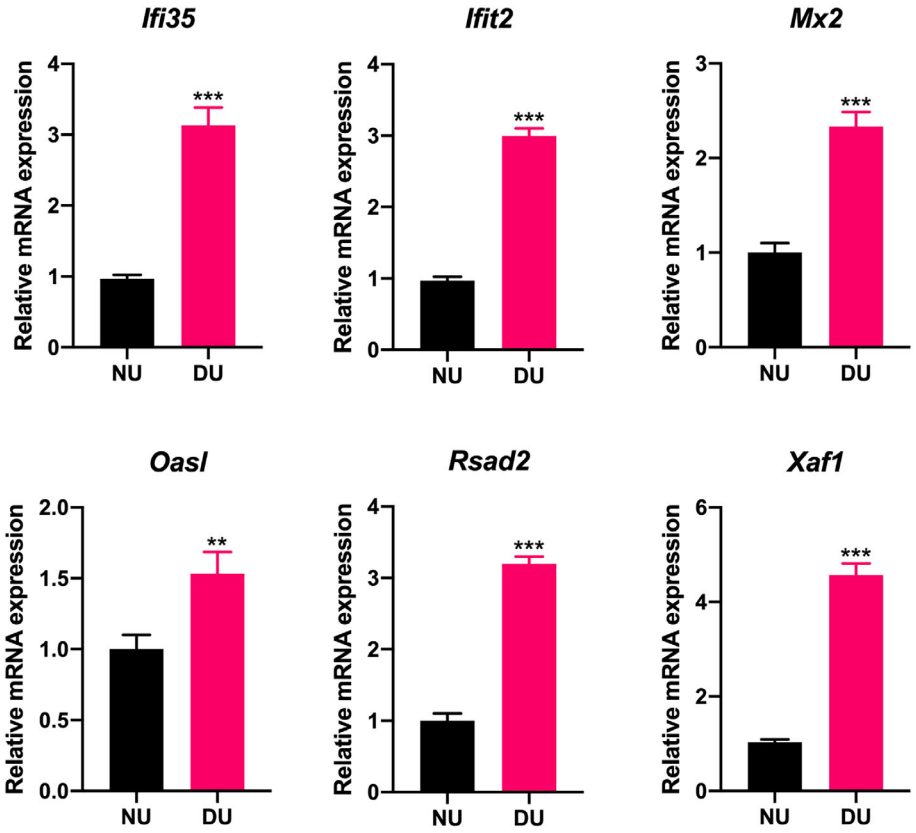

FIGURE 8 Experimental validation of the hub genes. mRNA expression levels of the hub differentially expressed genes detected by quantitative real-time polymerase chain reaction.
$^{**}P < .01$, $^{***}P < .001$

(2′-5′-Oligoadenylate Synthetase Like) was shown high expression in psoriasis. And 5 hub genes including OASL were overexpressed in TNF-α stimulated HaCaT cells.[46] Moreover, OASL was up-regulated in a variety of host cell types after infection of multiple gram-positive, gram-negative, and acid-resistant bacteria.[47] 40%–80% of DUs are co-infected, and the pathogens of infection are mainly gram-positive and gram-negative bacteria.[48] Radical S-Adenosyl Methionine Domain Containing 2 (RSAD2) inhibits the replication of a variety of viruses including Influenza Virus, Hepatitis C Virus, and chikungunya virus.[49-51] It plays a role in $CD4^+$ T-cells activation and differentiation and facilitates T-cell receptor-mediated GATA3 activation and optimal Th2 cytokine production by regulating NFκB1 and JUNB activities.[52] The deficiency of RSAD2 promotes polarisation of macrophages and secretion of M1 and M2 cytokines, suggesting that the expression of RSAD2 may play a critical regulatory role during macrophage polarisation.[53] Moreover, RSAD2 was up-regulated in M1-type macrophages.[54] MX2 (MX Dynamin Like GTPase 2) acts on antiviral defence, immunity, innate immunity, mRNA transport, and protein transport.[55] A study by Melissa et al. investigating MX2 potency to inhibit HIV-1 suggested that MX2 inhibits HIV-1 infection by inhibiting capsid-dependent nuclear import of subviral complexes.[56] And MX2 have important antihepatitis B virus (HBV) effector functions.[57] There is a report that the expression of MX2 increased in LPS-induced uveitis mice and was suppressed by dexamethasone (DEX).[58]

Similarly, XIAP Associated Factor 1 (XAF1) seems to function as a negative regulator of members of the inhibitor of apoptosis protein (IAP) family. It was noted as the hub genes identified by PCR. Some researcher has reported that XAF1 down-regulation may contribute to prostate cancer and gastric cancer development.[59,60] And it was confirmed as a key regulatory gene in skin development, and cutaneous wound healing.[61] The immune checkpoints, immune cell infiltration, and immune score demonstrated that immune response plays an important role in DUs. Our findings demonstrate that the expression level of M2 in DUs was much lower than in healthy tissue. Nevertheless, little difference was found in the M1 expression of the two groups. Recently, it has been shown that macrophage polarisation plays an important role in the diabetic wound healing process, mainly including two broad categories: classically activated macrophages (M1) and alternatively macrophages (M2).[62,63] M1 macrophages are associated with pro-inflammation, whereas M2 macrophages are associated with the production and secretion of anti-inflammatory cytokines, thereby alleviating the inflammatory response.[64,65] Typically, the current study indicates that the impaired M2 polarisation of macrophages in diabetic wounds is associated with

impaired wound closure, poor angiogenesis, and decreased collagen deposition.[66]

Taken together, our results suggest that IFI35, IFIT2, OASL, RSAD2, MX2, and XAF1 as DUs-associated hub genes that can help to explain the mechanism of DUs. There are still some limitations in the study. First, we only compared the mRNA expression of hub genes between DUs and healthy samples in mice. However, the intricate mechanisms and functions of the hub genes and immune cells need to be further validated in vitro. Second, achieving practical strategies for translating DUs risk-associated genetic variants into functional annotations and clinical applications remains further research.

5 | CONCLUSION

In summary, we got the key genes by WGCNA, PPI, and PCR after obtaining the diabetic ulcer gene sets from the datasets GSE37265 and GSE80178. GO terms, KEGG analysis and immune cell infiltration, and immune scores showed that biological functions are closely related to immune response. Moreover, the hub genes include IFI35, IFIT2, MX2, OASL, RSAD2, and XAF1. And they were highly expressed in DUs mice. Further experiments plan to prove the functions of these genes and the exact mechanism in DUs.

AUTHOR CONTRIBUTIONS

Zhan Zhang, Ying Zhang, and Dan Yang contributed equally. Jingsi Jiang and Le Kuai conceived and designed the study. Yue Luo, Ying Luo, and Yi Ru performed data curation. Jiankun Song, Xiaoya Fei, and Yiran Chen performed experimental work. Zhan Zhang, Ying Zhang, and Dan Yang prepared the original draft. Bin Li, Jingsi Jiang, and Le Kuai reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

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

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REFERENCES

1. Pearson-Stuttard J, Buckley J, Cicek M, Gregg EW. The changing nature of mortality and morbidity in patients with diabetes. *Endocrinol Metab Clin North Am*. 2021;50(3):357-368. doi:10.1016/j.ecl.2021.05.001
2. Saeedi P, Karuranga S, Hammond L, et al. Cardiovascular diseases and risk factors knowledge and awareness in people with type 2 diabetes mellitus: a global evaluation. *Diabetes Res Clin Pract*. 2020;165:108194. doi:10.1016/j.diabres.2020.108194
3. Guo Y, Wang G, Jing J, et al. Stress hyperglycemia may have higher risk of stroke recurrence than previously diagnosed diabetes mellitus. *Aging*. 2021;13(6):9108-9118. doi:10.18632/aging.202797
4. Forster RB, Garcia ES, Sluiman AJ, et al. Retinal venular tortuosity and fractal dimension predict incident retinopathy in adults with type 2 diabetes: the Edinburgh type 2 diabetes study. *Diabetologia*. 2021;64(5):1103-1112. doi:10.1007/s00125-021-05388-5
5. Pop-Busui R, Boulton AJ, Feldman EL, et al. Diabetic neuropathy: a position statement by the American Diabetes Association. *Diabetes Care*. 2017;40(1):136-154. doi:10.2337/dc16-2042
6. Armstrong DG, Boulton AJM, Bus SA. Diabetic foot ulcers and their recurrence. *N Engl J Med*. 2017;376(24):2367-2375. doi:10.1056/NEJMra1615439
7. Chamberlain RC, Kelly F, Wild Sarah H, et al. Foot ulcer and risk of lower limb amputation or death in people with diabetes: a National Population-Based Retrospective Cohort Study. *Diabetes Care*. 2022;45(1):83-91. doi:10.2337/dc21-1596
8. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. *Sci Transl Med*. 2014;6(265):265sr6. doi:10.1126/scitranslmed.3009337
9. Dinh T, Tecilazich F, Kafanas A, et al. Mechanisms involved in the development and healing of diabetic foot ulceration. *Diabetes*. 2012;61:2937-2947. doi:10.2337/db12-0227
10. Drela E, Stankowska K, Kulwas A, Rość D. Endothelial progenitor cells in diabetic foot syndrome. Adv. *Clin Exp Med*. 2012;21(2):249-254.
11. Mirza RE, Fang MM, Ennis WJ, Koh TJ. Blocking interleukin-1β induces a healing-associated wound macrophage phenotype and improves healing in type 2 diabetes. *Diabetes*. 2013;62(7):2579-2587. doi:10.2337/db12-1450
12. Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med*. 1999;341(10):738-746. doi:10.1056/NEJM199909023411006
13. Hanses F, Park S, Rich J, Lee JC. Reduced neutrophil apoptosis in diabetic mice during staphylococcal infection leads to

prolonged Tnfα production and reduced neutrophil clearance. *PLoS One*. 2011;6(8):e23633. doi:10.1371/journal.pone.0023633

14. Karima M, Kantarci A, Ohira T, et al. Enhanced superoxide release and elevated protein kinase C activity in neutrophils from diabetic patients: association with periodontitis. *J Leukoc Biol*. 2005;78(4):862-870. doi:10.1189/jlb.1004583
15. Wong SL, Demers M, Martinod K, et al. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat Med*. 2015;21(7):815-819. doi:10.1038/nm.3887
16. He M, Kubo H, Morimoto K, et al. Receptor for advanced glycation end products binds to phosphatidylserine and assists in the clearance of apoptotic cells. *EMBO Rep*. 2011;12(4):358-364. doi:10.1038/embor.2011.28
17. Friggeri A, Banerjee S, Biswas S, et al. Participation of the receptor for advanced glycation end products in efferocytosis. *J Immunol*. 2011;186(11):6191-6198. doi:10.4049/jimmunol.1004134
18. Zhu Y, Wang Y, Jia Y, Xu J, Chai Y. Roxadustat promotes angiogenesis through HIF-1α/VEGF/VEGFR2 signaling and accelerates cutaneous wound healing in diabetic rats. *Wound Repair Regen*. 2019;27(4):324-334. doi:10.1111/wrr.12708
19. Gallagher KA, Liu ZJ, Xiao M, et al. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. *J Clin Invest*. 2007;117(5):1249-1259. doi:10.1172/JCI29710
20. Oulas A, Minadakis G, Zachariou M, Sokratous K, Bourdakou MM, Spyrou GM. Systems bioinformatics: increasing precision of computational diagnostics and therapeutics through network-based approaches. *Brief Bioinform*. 2019;20(3):806-824. doi:10.1093/bib/bbx151
21. Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest*. 1991;87(2):432-438. doi:10.1172/JCI115014
22. Tessari P, Cecchet D, Cosma A, et al. Nitric oxide synthesis is reduced in subjects with type 2 diabetes and nephropathy. *Diabetes*. 2010;59(9):2152-2159. doi:10.2337/db09-1772
23. Schaper NC, Van Netten JJ, Apelqvist J, Lipsky BA, Bakker K, International Working Group on the Diabetic Foot (IWGDF). Prevention and management of foot problems in diabetes: a summary guidance for daily practice 2015, based on the IWGDF guidance documents. *Diabetes Res Clin Pract*. 2017;124:84-92. doi:10.1016/j.diabres.2016.12.007
24. Rong Y, Yang H, Xu H, et al. Bioinformatic analysis reveals hub immune-related genes of diabetic foot ulcers. *Front Surg*. 2022;5(9):878965. doi:10.3389/fsurg.2022.878965
25. Nguyen TB, Do DN, Nguyen-Thanh T, Tatipamula VB, Nguyen HT. Identification of five hub genes as key prognostic biomarkers in liver cancer via integrated bioinformatics analysis. *Biology (Basel)*. 2021;10(10):957. doi:10.3390/biology10100957
26. Jiang C, Liu Y, Wen S, Xu C, Gu L. In silico development and clinical validation of novel 8 gene signature based on lipid metabolism related genes in colon adenocarcinoma. *Pharmacol Res*. 2021;169:105644. doi:10.1016/j.phrs.2021.105644
27. Bakker K, Apelqvist J, Lipsky BA, Van Netten JJ, International Working Group on the Diabetic Foot. The 2015 IWGDF guidance documents on prevention and management of foot problems in diabetes: development of an evidence-based global consensus. *Diabetes Metab Res Rev*. 2016;32(Suppl 1):2-6. doi:10.1002/dmrr.2694
28. Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A. Inflammation and neuropeptides: the connection in diabetic wound healing. *Expert Rev Mol Med*. 2009;13(11):e2. doi:10.1017/S1462399409000945
29. Falanga V. Wound healing and its impairment in the diabetic foot. *Lancet*. 2005;366(9498):1736-1743. doi:10.1016/S0140-6736(05)67700-8
30. Hesketh M, Sahin KB, West ZE, Murray RZ. Macrophage phenotypes regulate scar formation and chronic wound healing. *Int J Mol Sci*. 2017;18(7):1545. doi:10.3390/ijms18071545
31. Kanno Y, Hirade K, Ishisaki A, et al. Lack of alpha2-antiplasmin improves cutaneous wound healing via over-released vascular endothelial growth factor-induced angiogenesis in wound lesions. *J Thromb Haemost*. 2006;4(7):1602-1610. doi:10.1111/j.1538-7836.2006.01978.x
32. De Masi R, Orlando S, Bagordo F, Grassi T. IFP35 is a relevant factor in innate immunity, multiple sclerosis, and other chronic inflammatory diseases: a review. *Biology (Basel)*. 2021;10(12):1325. doi:10.3390/biology10121325
33. Yu Y, Xu N, Cheng Q, et al. IFP35 as a promising biomarker and therapeutic target for the syndromes induced by SARS-CoV-2 or influenza virus. *Cell Rep*. 2021;37(12):110126.
34. Das A, Dinh P, Panda D, Pattnaik AK. Interferon-inducible protein IFI35 negatively regulates RIG-I antiviral signaling and supports vesicular stomatitis virus replication. *J Virol*. 2014;88(6):3103-3113.
35. Gounder A, Yokoyama C, Jarjour N, Bricker T, Edelson B, Boon AC. Interferon induced protein 35 exacerbates H5N1 influenza disease through the expression of IL-12p40 homodimer. *PLoS Pathogens*. 2018;14(4):e1007001.
36. Xiahou Z, Wang X, Shen J, et al. NMI and IFP35 serve as proinflammatory DAMPs during cellular infection and injury. *Nat Commun*. 2017;8(1):950. doi:10.1038/s41467-017-00930-9
37. Bandman O, Coleman RT, Loring JF, Seilhamer JJ, Cocks BG. Complexity of inflammatory responses in endothelial cells and vascular smooth muscle cells determined by microarray analysis. *Ann N Y Acad Sci*. 2002;975:77-90. doi:10.1111/j.1749-6632.2002.tb05943.x
38. Jian D, Wang W, Zhou X, et al. Interferon-induced protein 35 inhibits endothelial cell proliferation, migration and re-endothelialization of injured arteries by inhibiting the nuclear factor-kappa B pathway. *Acta Physiol*. 2018;223(3):e13037. doi:10.1111/apha.13037
39. Das Sarma J, Burrows A, Rayman P, et al. Ifit2 deficiency restricts microglial activation and leukocyte migration following murine coronavirus (m-CoV) CNS infection. *PLoS Pathogens*. 2020;16(11):e1009034.
40. Tran V, Ledwith M, Thamamongood T, et al. Influenza virus repurposes the antiviral protein IFIT2 to promote translation of viral mRNAs. *Nat Microbiol*. 2020;5(12):1490-1503.
41. Lai K, Hong Z, Hsieh J, et al. IFIT2-depleted metastatic oral squamous cell carcinoma cells induce muscle atrophy and cancer cachexia in mice. *J Cachexia Sarcopenia Muscle*. 2022;13(2):1314-1328.
42. Jia H, Song L, Cong Q, et al. The LIM protein AJUBA promotes colorectal cancer cell survival through suppression of JAK1/-STAT1/IFIT2 network. *Oncogene*. 2017;36(19):2655-2666.

43. Aung HT, Schroder K, Himes SR, et al. LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. *FASEB J*. 2006;20(9):1315-1327. doi:10.1096/fj.05-5360com
44. Siegfried A, Berchtold S, Manncke B, et al. IFIT2 is an effector protein of type I IFN-mediated amplification of lipopolysaccharide (LPS)-induced TNF-α secretion and LPS-induced endotoxin shock. *J Immunol*. 2013;191(7):3913-3921. doi:10.4049/jimmunol.1203305
45. Huang C, Lewis C, Borg NA, et al. Proteomic identification of interferon-induced proteins with tetratricopeptide repeats as markers of M1 macrophage polarization. *J Proteome Res*. 2018;17(4):1485-1499. doi:10.1021/acs.jproteome.7b00828
46. Gao LJ, Shen J, Ren YN, Shi JY, Wang DP, Cao JM. Discovering novel hub genes and pathways associated with the pathogenesis of psoriasis. *Dermatol Ther*. 2020;33(6):e13993. doi:10.1111/dth.13993
47. Leisching G, Ali A, Cole V, Baker B. 2'-5'-Oligoadenylate synthetase-like protein inhibits intracellular *M. tuberculosis* replication and promotes proinflammatory cytokine secretion. *Mol Immunol*. 2020;118(73–78):73-78. doi:10.1016/j.molimm.2019.12.004
48. Goh TC, Bajuri MY, Nadarajah SC, Abdul Rashid AH, Baharuddin S, Zamri KS. Clinical and bacteriological profile of diabetic foot infections in a tertiary care. *J Foot Ankle Res*. 2020;13(1):36. doi:10.1186/s13047-020-00406-y
49. Wang X, Hinson E, Cresswell P. The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. *Cell Host Microbe*. 2007;2(2):96-105.
50. Helbig K, Eyre N, Yip E, et al. The antiviral protein viperin inhibits hepatitis C virus replication via interaction with nonstructural protein 5A. *Hepatology*. 2011;54(5):1506-1517.
51. Teng T, Foo S, Simamarta D, et al. Viperin restricts chikungunya virus replication and pathology. *J Clin Investig*. 2012;122(12):4447-4460.
52. Sherrill JD, Kc K, Wang X, et al. Whole-exome sequencing uncovers oxidoreductases DHTKD1 and OGDHL as linkers between mitochondrial dysfunction and eosinophilic esophagitis. *JCI Insight*. 2018;3(8):e99922. doi:10.1172/jci.insight.99922
53. Eom J, Yoo J, Kim JJ, et al. Viperin deficiency promotes polarization of macrophages and secretion of M1 and M2 cytokines. *Immune Netw*. 2018;18(4):e32. doi:10.4110/in.2018.18.e32
54. Li P, Hao Z, Wu J, et al. Comparative proteomic analysis of polarized human THP-1 and mouse RAW264.7 macrophages. *Front Immunol*. 2021;29(12):700009. doi:10.3389/fimmu.2021.700009
55. Diotallevi M, Checconi P, Palamara AT, et al. Glutathione fine-tunes the innate immune response toward antiviral pathways in a macrophage cell line independently of its antioxidant properties. *Front Immunol*. 2017;29(8):1239. doi:10.3389/fimmu.2017.01239
56. Kane M, Yadav S, Bitzegeio J, et al. MX2 is an interferon-induced inhibitor of HIV-1 infection. *Nature*. 2013;502(7472):563-566.
57. Wang Y, Niklasch M, Liu T, et al. Interferon-inducible MX2 is a host restriction factor of hepatitis B virus replication. *J Hepatol*. 2020;72(5):865-876.
58. Yu P, Qiu Y, Lin R, Fu X, Hao B, Lei B. Retinal transcriptome profile in mice following dexamethasone treatment for endotoxin-induced uveitis. *J South Med Univ*. 2018;38(8):901-909. doi:10.3969/j.issn.1673-4254.2018.08.01
59. Jeong S, Kim J, Ko K, et al. XAF1 forms a positive feedback loop with IRF-1 to drive apoptotic stress response and suppress tumorigenesis. *Cell Death Dis*. 2018;9(8):806.
60. Xing Z, Zhou Z, Yu R, et al. XAF1 expression and regulatory effects of somatostatin on XAF1 in prostate cancer cells. *J Exp Clin Cancer Res*. 2010;29:162.
61. Ashrafi M, Sebastian A, Shih B, et al. Whole genome microarray data of chronic wound debridement prior to application of dermal skin substitutes. *Wound Repair Regen*. 2016;24(5):870-875. doi:10.1111/wrr.12460
62. Feng J, Dong C, Long Y, et al. Elevated Kallikrein-binding protein in diabetes impairs wound healing through inducing macrophage M1 polarization. *Cell Commun Signal*. 2019;17(1):60.
63. He R, Yin H, Yuan B, et al. IL-33 improves wound healing through enhanced M2 macrophage polarization in diabetic mice. *Mol Immunol*. 2017;90:42-49.
64. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol*. 2002;23(11):549-555.
65. Kelly B, O'Neill LA. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res*. 2015;25:771-784.
66. Kim H, Wang SY, Kwak G, Yang Y, Kwon IC, Kim SH. Exosome-guided phenotypic switch of M1 to M2 macrophages for cutaneous wound healing. *Adv Sci*. 2019;6(20):1900513.

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