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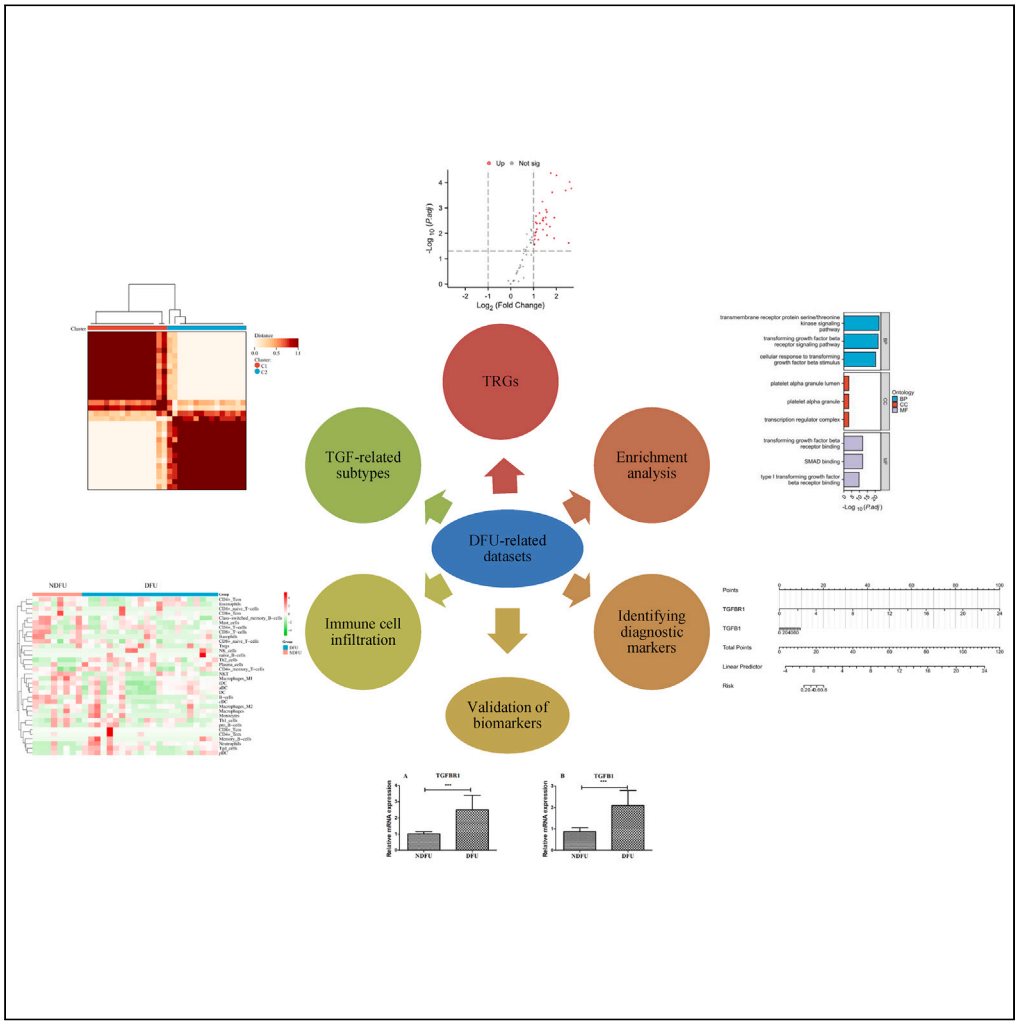

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Highlights

TGF-β signaling pathway is activated in DFU

TGFBR1 and TGFB1 as potential markers for the DFU

Two TGF-related patterns with differing biological functions and immune infiltration

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Bioinformatics analysis identifies TGF-β signaling pathway-associated molecular subtypes and gene signature in diabetic foot

Guanggang Du,[1,2,4] Jie Chen,[1,2,4] Xuezhu Zhu,[1,2,*] and Zongdong Zhu[2,3,5,*]

SUMMARY

The role of transforming growth factor β (TGF-β) in inflammation and immune response is established, but the mechanism of TGF-β signaling pathway-related genes (TRGs) in diabetic foot ulcer (DFU) is not fully understood. We aimed to investigate the contribution of TRGs in the identification, molecular categorization, and immune infiltration of DFU through bioinformatics analysis. TGF-β signaling pathway is activated in DFU. 33 TRGs were upregulated. Regression analysis revealed TGFBR1 and TGFB1 as significant differential expression core genes, validated by quantitative real-time PCR. The diagnostic model with core genes had high clinical validity (AUC = 0.909). Core gene expression was associated with immune cell infiltration. A total of 5672 genes showed differential expression in TGF-related patterns, with differences in biological functions and immune infiltration. TGF-β signaling pathway may be critical in DFU development.

INTRODUCTION

Diabetic foot ulcer (DFU) is serious complication of diabetes.[1] It is a major contributor to morbidity and mortality in diabetic patients, and can be both complicated and expensive to treat.[2] The lifetime risk of developing a foot ulcer for a diabetic patient is estimated to be between 19% and 34%.[3] Furthermore, the rates of recurrence for ulcers remain alarmingly high, with 40% of patients experiencing a recurrence within a year after healing, and 65% within five years.[3] Among US adults in 2016, there were 130,000 hospital discharges resulting from lower extremity amputations caused by DFU. This highlights the significant financial burden that DFU-related healthcare costs pose globally.[4] The course of DFU is often worsened by extensive diabetic alterations, including neuropathy and vascular disease.[5] Although there have been notable improvements in the management of DFU in recent times, a considerable number of cases still progress into chronic wounds via an irreversible process.[6,7] Therefore, it is crucial to explore the molecular mechanisms of DFU to enhance treatment efficiency and prognosis for patients with DFU.

The progression of DFU is a complex process influenced by various intrinsic factors. These factors include vascular problems, fibrosis, impaired immune function, infection, ischemia, and neuropathy. The presence of these factors contributes to poor healing outcomes in patients with DFU.[8–10] The inflammatory response is a vital initial response that occurs after an injury, aiding in the healing process. This process involves attracting immune cells to the site of injury, which coordinates a multicellular healing response and helps prevent infections from occurring.[11–13] Transforming growth factor β (TGF-β) isoforms are pleiotropic cytokines that are highly homologous and are produced by various leukocyte subsets such as dendritic cells, monocytes, natural killer cells, mast cells, neutrophils, B cells, and T cells, as well as platelets found in both circulation and tissues.[14] TGF-β plays a critical role in maintaining immune homeostasis and tolerance by suppressing the growth and activity of various immune system components.[15] Any disruptions in TGF-β signaling can lead to inflammatory disorders and facilitate the development of tumors.[16] TGF-β1 plays a significant role in the wound healing process. It is primarily stored in platelets, which release it during the acute response to injury.[17] In addition, TGF-β is widely acknowledged as a crucial mediator in the development of diabetic nephropathy.[18] A recent study has suggested that drugs targeting the TGF-β signaling pathway and its blockade have produced promising results in the management of diabetes and its associated complications.[19] These studies have highlighted the importance of TGF-β signaling in various diseases, including diabetes, but its specific role in DFU remains unclear. Therefore, this study fills a knowledge gap by exploring the relationship between TGF-β signaling pathway and DFU.

The primary research question of our study is whether there are distinct molecular subtypes and signature genes associated with the TGF-β signaling pathway in DFU patients. We hypothesized that the activation or dysregulation of the TGF-β signaling pathway would lead to the formation of different molecular subtypes and gene expression patterns, which may contribute to the development and progression of DFU.

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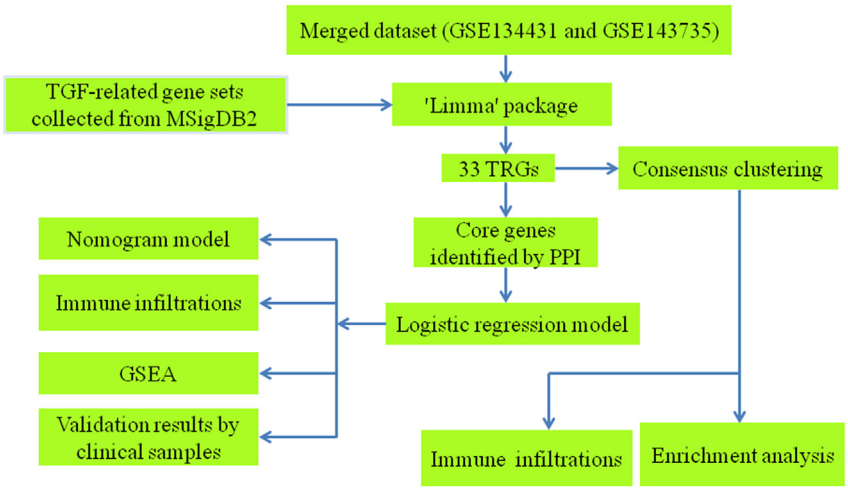

Figure 1. Study workflow diagram

The integration of molecular biology and information technology has given rise to the field of bioinformatics, which has allowed for a new understanding of diseases at the genetic level.[20] As far as we are aware, there has been a lack of extensive research on the discovery of potential diagnostic candidates associated with TGF-β signaling for DFU, as well as the utilization of bioinformatics in DFU diagnosis.

In this study, we utilized data from bulk RNA sequencing to conduct an integrative analysis and identify the core genes of differentially expressed TGF-β signaling pathway-related genes (DETRGs) in DFU. We also developed and validated a diagnostic model for DFU. Furthermore, we used unsupervised cluster analysis to divide DFU samples into different molecular subtypes based on these DETRGs. This allowed us to investigate the changes in the enrichment pathways and level of immune cell infiltration among the different molecular subtypes. Our research presents novel contributions, utilizing bioinformatics to uncover molecular subtypes and gene signatures linked to TGF-β in DFU, enabling a detailed analysis of the disease's molecular intricacies. Moreover, we clarify the particular molecular processes of DFU related to TGF-β, which could lead to new targeted treatments and precision medicine for affected individuals.

RESULTS

The landscape of TRGs between NDFU and DFU samples

Figure 1 shows the study flowchart. After removing the batch effect between two datasets related to DFUs (GSE134431 and GSE143735) (Table 1), we created an integrated database consisting of 8 non-DFU (NDFU) samples and 22 DFU samples with gene expression profiles. As shown in Figure 2A, we first performed a gene set enrichment analysis (GSEA) on the integrated dataset, the results of which showed that the TGF-β signaling pathway was significantly enriched in the DFU group ($p = 0.0018$). The gene set variation analysis (GSVA) algorithm was used to calculate the TGF score and it was found that the NDFU group had a significantly lower score in comparison to the DFU group (Figure 2B, $p < 0.001$). To compare the expression levels of differentially expressed TRGs between NDFU and DFU samples, a volcano plot and heatmap were generated. As shown in Figures 2C and 2D, the 33 DETRGs were found to be upregulated in the DFU group compared with those in the NDFU group (Table S3). These findings suggested that TGF-β signaling pathway may be a significant factor in the advancement of DFU.

Protein-protein interaction network and functional enrichment analysis of DETRGs

Among these DETRGs, most of them showed close interactions with each other (Figure 3A). As shown in Figure 3B, the enrichment analysis results for GO-BP mainly involved transmembrane receptor protein serine kinase signaling pathway, TGF-β receptor signaling pathway, etc.; for the GO-CC analysis, the enrichment pathways were mainly involved in platelet alpha granule lumen, transcription regulator complex, etc.; for the GO-MF analysis, the enrichment pathways were mainly involved in TGF-β receptor binding, SMAD binding, etc. Based on the results of KEGG analysis, the top 10 enrich pathways were mainly enriched in TGF-beta signaling pathway, Chagas disease, cellular senescence, chronic myeloid leukemia, AGE-RAGE signaling pathway in diabetic complications, etc (Figure 3C). Therefore, due to the similarity of their gene disorders, the pathological mechanisms of DFU may coincide with those found in certain infectious and metabolic disorders. In addition, the

Table 1. Basic information of GEO datasets

GEO ID	Platform	NDFU	DFU	Source
GSE134431	GPL18573	8	13	Foot skin
GSE143735	GPL11154	0	9	Forearm whole skin

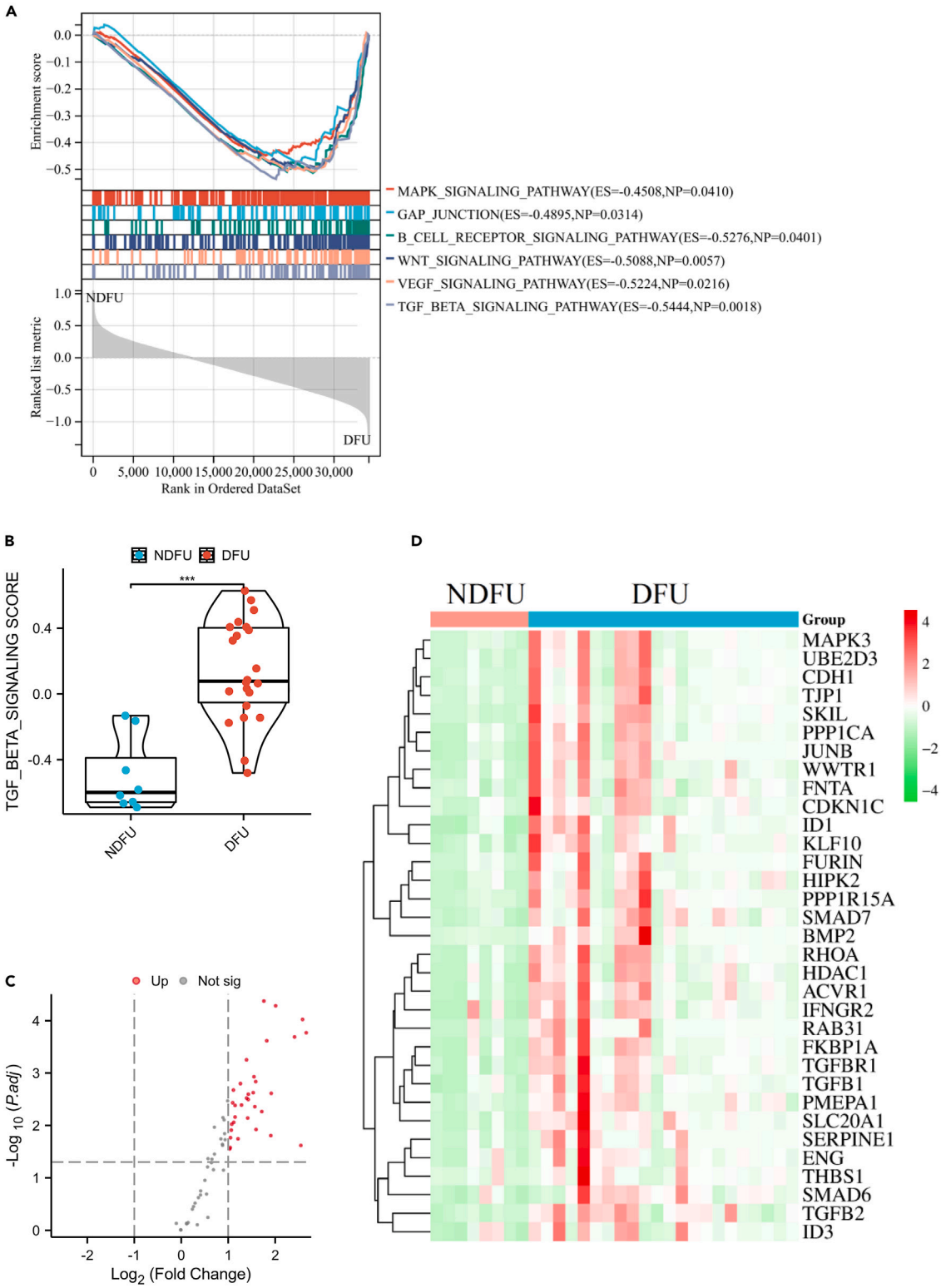

Figure 2. Differential expression of TRGs in DFU

(A) The results of GSEA between NDFU and DFU groups.

(B) Violin plot presented the TGF score levels differing between the DFU and NDFU groups. Volcano plot (C) and heatmap (D) of DETRGs between NDFU and DFU groups. Statistical comparison between the two groups was performed using the Wilcoxon rank-sum test. ***p < 0.001.

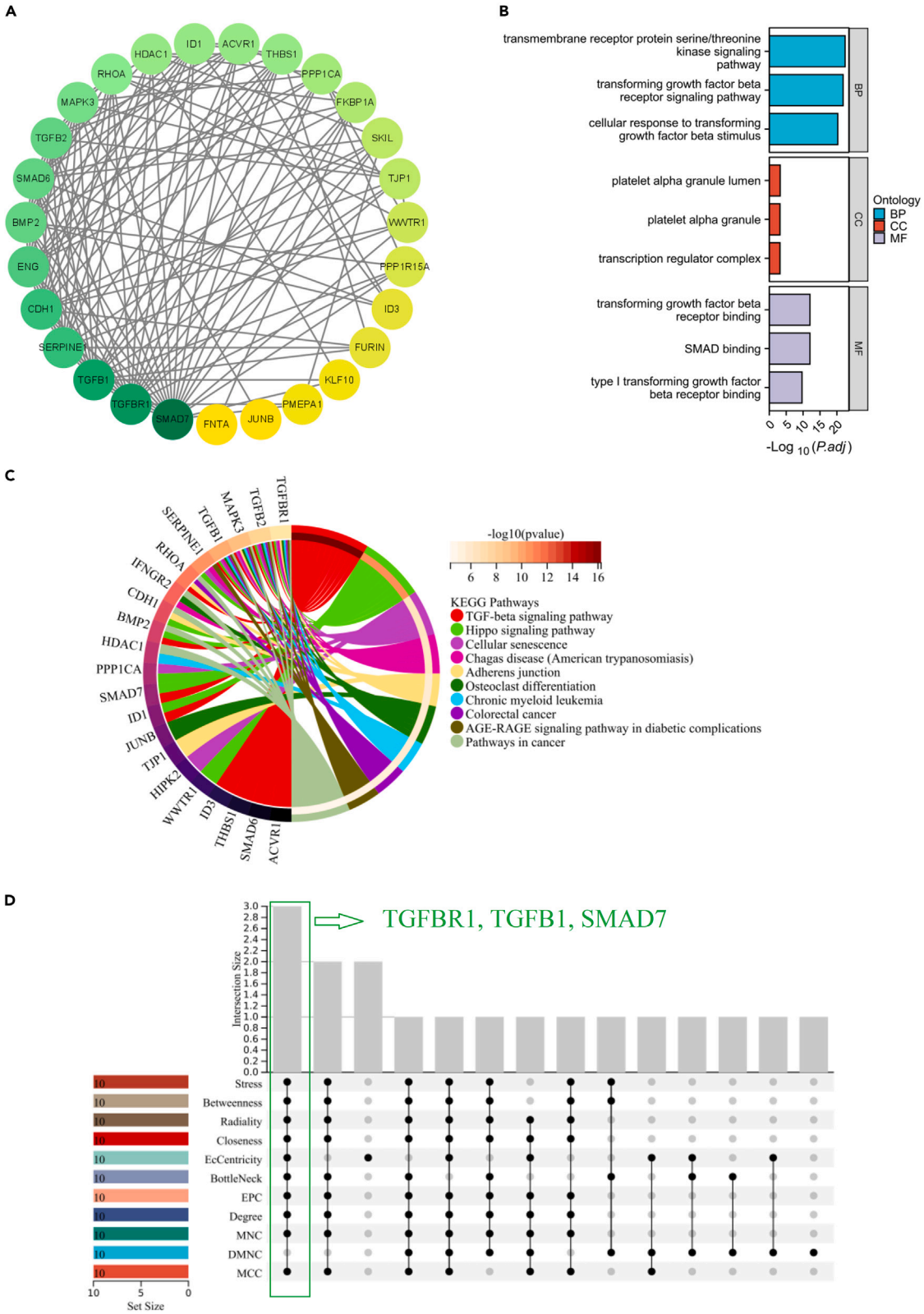

Figure 3. PPI network and functional enrichment analysis of DETRGs

(A) PPI network of 33 DETRGs. The results of GO (B) and KEGG (C) enrichment analysis of 33 DETRGs. (D) Core genes were identified by cytoHubba plug-in.

Table 2. Logistic regression identifies diagnostic markers for DFU patients

		Univariate analysis		Multivariate analysis	
Characteristics	Total(N)	Odds Ratio (95% CI)	p value	Odds Ratio (95% CI)	p value
TGFBR1	30	3.217 (1.347–7.682)	**0.009**	2.928 (0.941–9.106)	0.064
TGFB1	30	1.277 (1.059–1.539)	**0.010**	1.033 (0.790–1.351)	0.812

TGF-β type 1 receptor (TGFBR1), TGFB1, and SMAD7 genes were identified based on the overlapped parameters of the top 10 DETRGs in 11 algorithms (Figure 3D).

The logistic regression analysis identifies diagnostic markers

A logistic regression analysis was performed with three TRGs (TGFBR1, TGFB1, and SMAD7), leading to the discovery of two hub genes (TGFBR1 and TGFB1) with p values less than 0.05 (Table 2). To improve the prediction of a patient developing DFU, we have developed a nomogram that considers the two diagnostic genes (Figure 4A). The diagnostic genes were upregulated in the DFU group compared with those in the NDFU group (Figure 4B). The model demonstrated an area under the curve (AUC) value of 0.909 through receiver operating characteristic analysis (Figure 4C). The results of the calibration curve indicated that the nomogram model has a high level of accuracy in predicting outcomes for patients with DFU (Figure 4D). Additionally, the decision curve analysis revealed that the utilization of the nomogram model could provide significant benefits for patients with DFU. The results consistently showed that the light blue line (representing the nomogram model) outperformed both the red line (representing all) and the deep green line (representing none) across the entire range from 0 to 0.9 (Figure 4E). These findings demonstrated that the signature genes associated with TGF-β signaling pathway possessed effective diagnostic significance for patients with DFU.

Differences in immune characteristics between NDFU and DFU

As shown in Figures 5A and 5B, xCell analysis indicated that DFU group had significantly higher levels of neutrophils, pDC, Tgd cells, and Th2 cells than those in NDFU group ($p < 0.05$). However, the levels of B cells, $CD4^+$ naive T cells, $CD8^+$ T cells, cDC, class-switched memory B cells, and NKT were significantly lower than those NDFU group ($p < 0.05$). In addition, TGFBR1 and TGFB1 were positively correlated with Tgd cells, neutrophils, pDC, and Th2 cells; TGFBR1 and TGFB1 had negatively correlated with $CD8^+$ T cells, class-switched memory B cells, and basophils (Figures 5C and 5D). These findings suggested a potential association between activated immune cells and the activation of TGF-β signaling pathway in DFU.

Single-gene GSEA

Single-gene GSEA was performed to explore the potential biological functions regulated by TGFBR1 and TGFB1 in DFU. Our findings showed that VEGF signaling pathway, P53 signaling pathway, WNT signaling pathway, MAPK signaling pathway, insulin signaling pathway, TGF-β signaling pathway, endocytosis, and lysosome were significantly enriched in the TGFB1-high subgroup and TGFBR1-high subgroup (Figures 6A and 6B).

Validation of diagnostic biomarkers by clinical samples

We collected clinical samples to validate the gene expression levels of diagnostic markers. As shown in Figure 7, TGFBR1 and TGFB1 gene expression levels were significantly upregulated in the DFU group compared to the NDFU group. This result was consistent with the bioinformatics results.

Identification of different TGF-related subtypes in DFU

In this study, we analyzed the expressions of 33 DETRGs and utilized unsupervised consensus clustering to categorize DFU patients based on their TRGs expressions. As a result, we identified two distinct subtypes (C1 and C2) of DFU samples (Figure 8).

Differential expression and enrichment analysis between the two subgroups

To compare the two subtypes, we identified a total of 5672 differentially expressed genes (DEGs) between C1 and C2. Out of these, 4829 DEGs were upregulated while 843 DEGs were downregulated in C2 (Figure 9A and Table S4). The heatmap analysis showed that these DEGs were able to distinguish between the two TGF subgroups (Figure 9B). GSEA results showed that these DEGs were enriched in oxidative phosphorylation, P53 signaling pathway, acute myeloid leukemia, lysosome, endocytosis, apoptosis, B cell receptor signaling pathway, insulin signaling pathway, WNT signaling pathway, VEGF signaling pathway, TGF-β signaling pathway, T cell receptor signaling pathway, MAPK signaling pathway, chemokine signaling pathway, natural killer cell-mediated cytotoxicity, etc (Figure 9C). GO enrichment analysis indicated that these DEGs were enriched in endosome organization, proton-transporting V-type ATPase complex, proton-transporting ATPase activity, etc (Figure 9D). KEGG pathways revealed that these DEGs were enriched in endocytosis, oxidative phosphorylation,

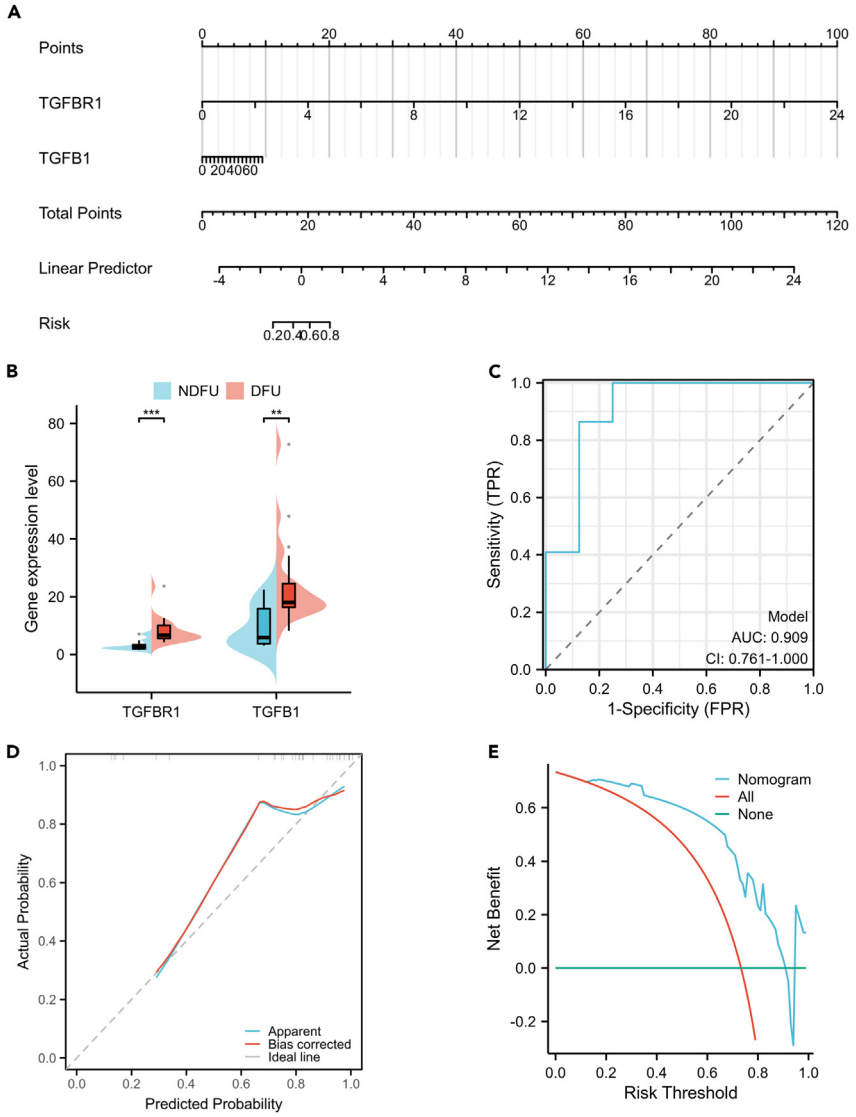

Figure 4. Nomogram model

(A) Nomogram plots of diagnostic model based on TGFB1 and TGFBR1 genes.

(B) Boxplot presented the expression levels of TGFB1 and TGFBR1. Statistical comparison between the two groups was performed using the Wilcoxon rank-sum test. **p < 0.01, ***p < 0.001.

(C) ROC curve of predicted risk score in DFU diagnosis.

(D) Calibration curve for the diagnostic model.

(E) Decision curve analysis based on the diagnostic model.

regulation of autophagy, apoptosis, and Alzheimer's disease (Figure 9E), which were consistent with the results of GSEA. In addition, GSVA results showed that acute myeloid leukemia, regulation of autophagy, MAPK signaling pathway, endocytosis, and VEGF signaling pathway were significantly up-regulated in the C2 subtype (Figures 10A and 10B), which were consistent with the results of GSEA. As a result, because of the resemblance of their gene disorders, the pathological mechanisms of the TGF-related subtype in DFU might be involved in immune and inflammation processes.

Differences between the two TGF subtypes in TRGs expression levels and immune environment characteristics

The analysis of TRGs showed that, with the exception of TGFB2, SMAD6, THBS1, ENG, SERPINE1, and ID3, the other 27 TRGs displayed significant variations in transcriptome profiles across each cluster (Figures 11A and 11B). These findings suggest that TGF plays a role in the regulation of DFU development.

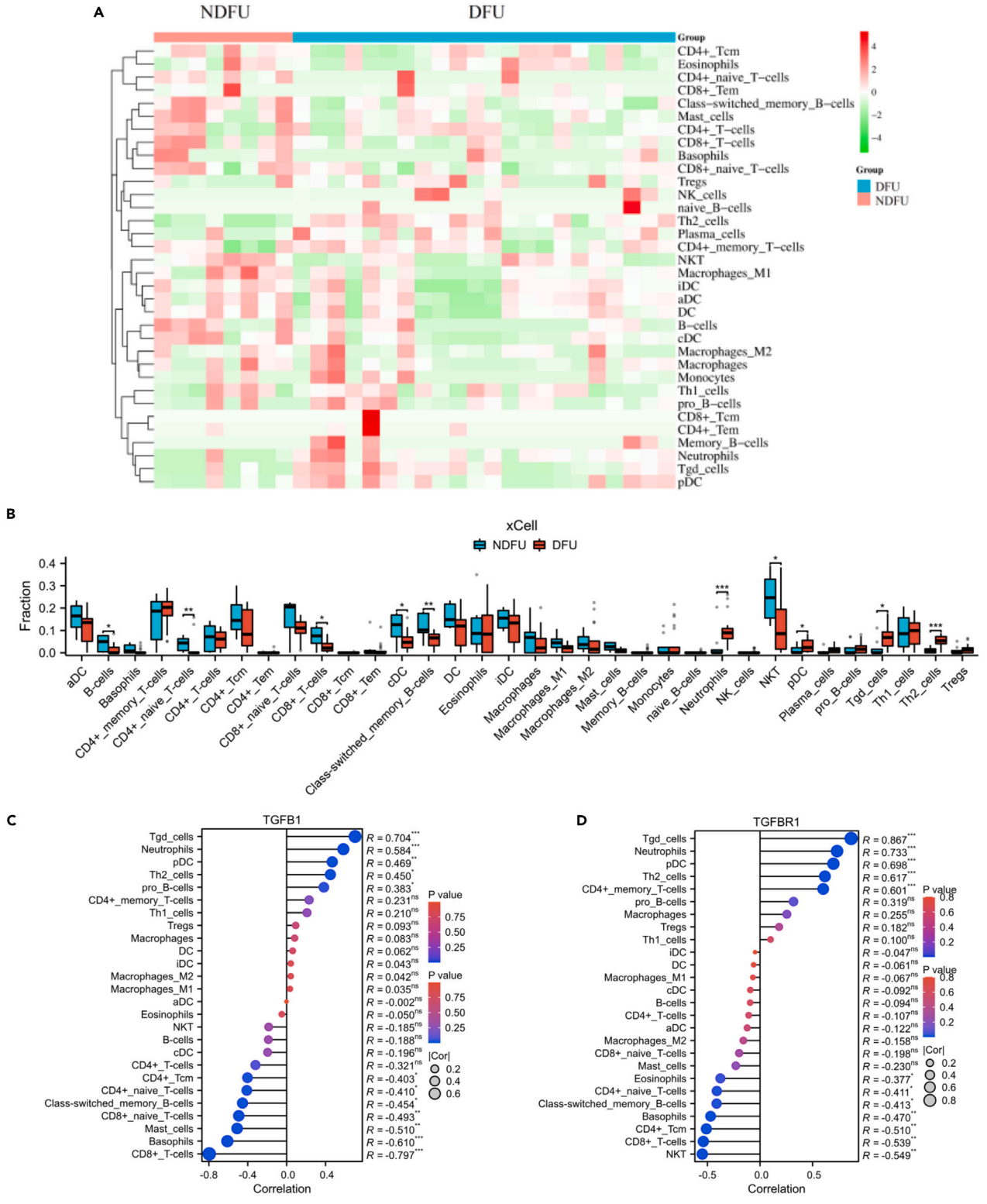

Figure 5. Landscape of the immune cell infiltrates in DFU

The heatmap (A) and boxplot (B) presented the differences in immune environment characteristics between the DFU and NDFU groups. Correlation analysis of the TGFB1 (C) and TGFBR1 (D) gene expression with immune infiltration. Statistical comparison between the two groups was performed using the Wilcoxon rank-sum test. *p < 0.05, **p < 0.01, ***p < 0.001.

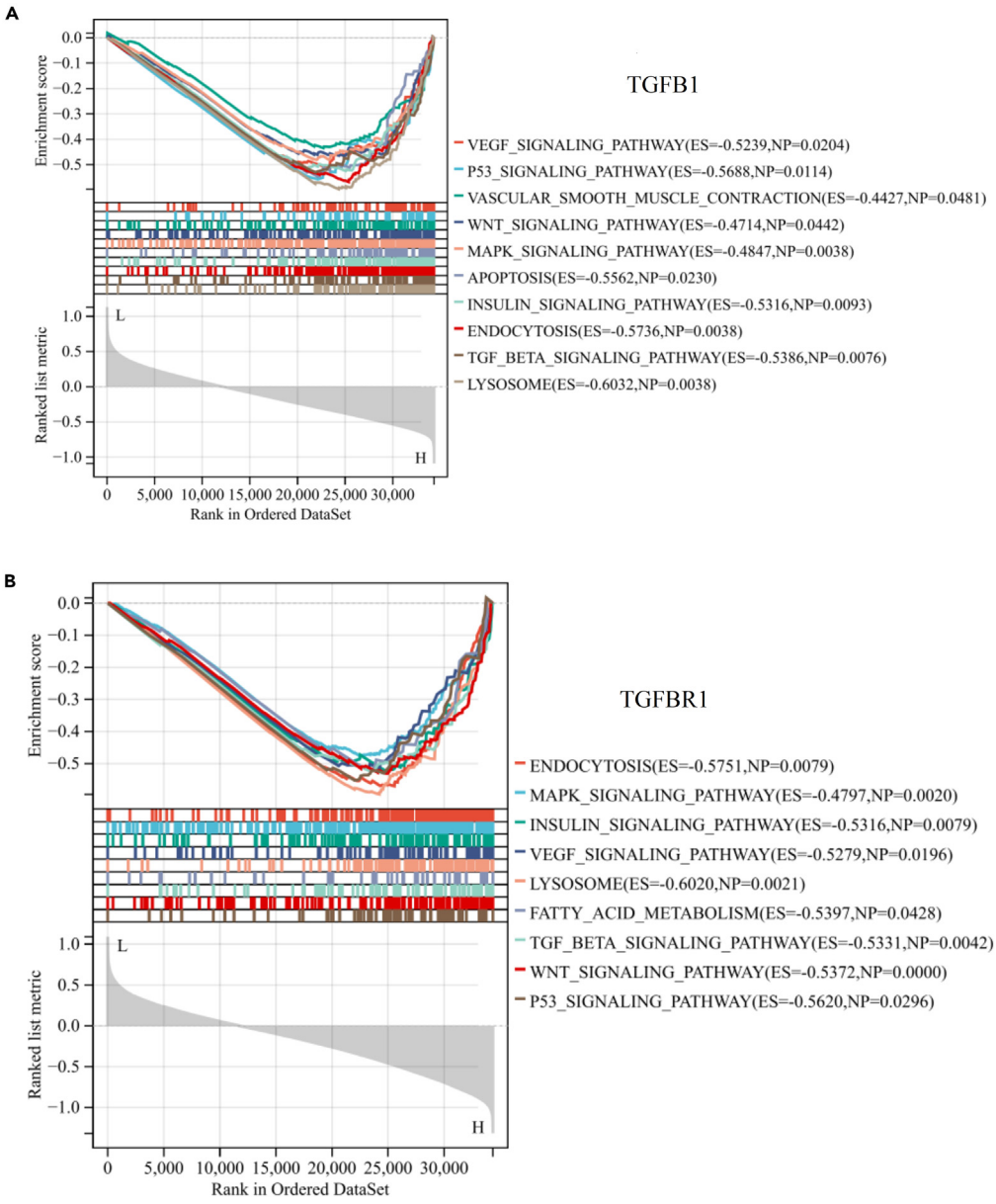

Figure 6. GSEA of trait genes in DFU

Single-gene GSEA of TGFB1 (A) and TGFBR1 (B).

As shown in Figure 11C, xCell analysis indicated that C2 subgroup had significantly higher levels of $CD4^+$ memory T cells, neutrophils, pDC, plasma cells, Tgd cells, and Th2 cells than those in C1 subgroup ($p < 0.05$). However, the levels of $CD4^+$ naive T cells, class-switched memory B cells, and NKT were significantly lower than those C1 subgroup ($p < 0.05$). In DFU patients, there was a differential infiltration of various types of immune cells in TGF-related subtypes. This suggested that these immune cells could potentially be targeted for regulating DFU treatment.

DISCUSSION

DFU is a significant cause of mortality in diabetic patients.[21] Therefore, it is imperative to identify molecular therapeutic targets that are specific to DFU to improve patient prognosis and reduce mortality rates. The TGF-β family is composed of numerous proteins that are secreted and have similar structures. These proteins have various functions such as developmental patterning, tissue differentiation, and maintaining homeostasis.[22] TGF-β has been demonstrated to regulate various steps involved in the wound healing process by influencing multiple cell types.[23] Previous studies showed a link between TGF and the development of DFU.[24,25] However, the role of TRGs on DFU remains unclear.

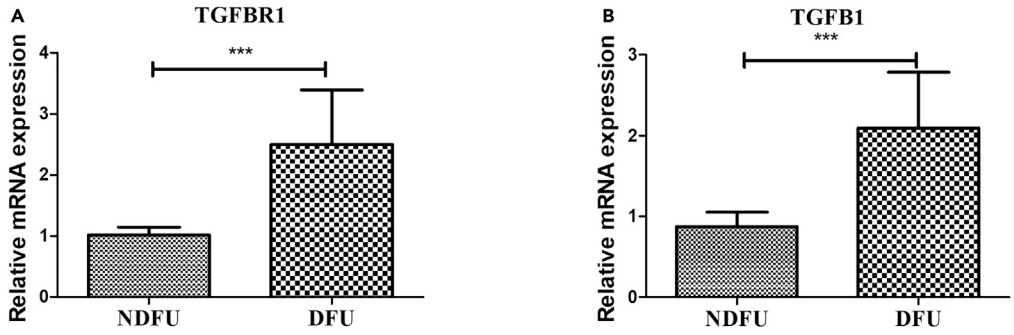

Figure 7. Validation of diagnostic biomarkers by clinical samples

Validation of TGFBR1 (A) and TGFB1 (B) by clinical samples. Statistical comparison between the two groups was performed using the Wilcoxon rank-sum test. ***$p < 0.001$.

Bioinformatics technology and microarray technology are the primary methods used to investigate gene expression levels. These approaches have significantly enhanced our comprehension of the underlying molecular mechanisms involved in complex disorders such as diabetes.[26,27] In this study, we employed integrated bioinformatics analysis and machine learning techniques to investigate the involvement of TGF-related genes in DFU. We conducted an analysis of TRGs expression profiles, identified prognostic signature genes, developed DFU risk models, evaluated immune infiltration, and performed clustering analysis. This is the first study to utilize these methods in examining the role of TGF-related genes in DFU.

Our study has revealed two significant TGF-associated candidate genes, namely TGFB1 and TGFBR1, which can aid in the diagnosis of DFU patients. Additionally, we have developed a nomogram that can be used as a tool to diagnose DFU from patients with diabetic foot. The diagnostic model utilized two biomarkers (TGFB1 and TGFBR1) that were found to be significant. The AUC of the model was greater than 0.9, indicating that the model has a high level of accuracy in distinguishing samples with DFU from those without. Based on the correlation with changes in the immune microenvironment, it is possible that TGFB1 and TGFBR1 are closely associated with both the diagnosis and immunotherapy of DFU. TGFB1 is a multifunctional cytokine that plays a vital role in cancer, fibrosis, and development.[28,29] The regulation of chemotaxis, activation, and survival of various immune cells is controlled by TGF-β. This control mechanism plays a crucial role in both the initiation and resolution of inflammatory responses.[30] TGF-β is a regulator of cell differentiation, migration, proliferation, and apoptosis in wound healing.[31] Additionally, the serum and dorsalis pedis arteries of DFU patients showed significantly higher levels of TGF-β1 protein and mRNA expression compared to the control group.[32] A previous study observed significant increases in TGF-β1 levels in the serum of individuals with both diabetic kidney disease and DFU, compared to control group,[25] which is consistent with our results. TGFBR1 serves as a crucial mediator for transmitting extracellular stimulation to the downstream TGF-β signaling pathway.[33] The dysfunction or loss of beta cells is the common pathological feature in all types of diabetes mellitus. TGFBR1 promotes the proliferation of beta cells after pharmacological inhibition.[34,35] The suppression of TGF-β receptors expression plays a crucial role in promoting regenerative wound healing and inhibiting scar formation.[36] Following a wound, the expression levels of TGFBR1 and TGFBR2 were found to be high in cells that migrate to cover a corneal wound.[37] Additionally, TGFBR1 may be involved in diabetic wound fibroblasts.[38] However, to date, there have been no reports of studies investigating the association between the two signature genes and DFU. Furthermore, there is a scarcity of clinical indicators available for diagnosing patients with DFU. Our study aims to fill this gap and potentially offers insights for future experimental investigations on these signature genes. Next, the correlation between signature genes and the infiltration of immune cells in patients with diabetic foot was examined, indicating the significant involvement of the TGF-β signaling pathway in regulating the immune microenvironment of diabetic foot.

During the progression of DFU, we found that TGFB1 and TGFBR1 are correlated with immune cell infiltrations. Diabetes predisposes patients to wound infection by impairing the immune response.[39] The recruitment of immune cells to the site of damage is essential for preventing infection and coordinating the multicellular healing response.[11,40] The first inflammatory cells to migrate to the wound are neutrophils, which play a vital role in the infectious microorganisms and clearance of dead cells.[41,42] In our study, we discovered that patients with diabetic foot can be categorized into two distinct subtypes based on the TRGs expression profiles. These subtypes exhibited varying levels of immune cell infiltration. Specifically, in the C1 subtypes, levels of neutrophils, pDC, Tgd cells, and Th2 cells were downregulated, whereas $CD4^+$ naive T cells and NKT were upregulated. Consistent with our findings, the process of "NETosis" in response to high glucose levels in diabetes leads to an excessive release of neutrophil extracellular traps, which in turn inhibits the natural process of wound healing.[43] Neutrophils from patients with diabetic foot ulcers exhibit spontaneous priming of NETosis, resulting in increased neutrophil extracellular traps and activation of the NLRP3 inflammasome. These factors have been linked to impaired wound healing.[44] Compared to the type 2 diabetes mellitus group, a significant decrease in NKT and IFN-γ was observed in the diabetic foot infection group.[45] Based on the aforementioned findings, we posit that the TGF-β signaling pathway plays a significant role in the progression of DFU by modulating the infiltration of immune cells.

In summary, our study highlighted the crucial involvement of TGF-β signaling pathway in the advancement and formation of DFU. Additionally, we have identified two core genes that could serve as potential biomarkers and targets for diagnosing DFU patients. These findings can serve as a reference for further understanding the pathogenesis of DFU and can aid in the development of drug screening and personalized therapy for DFU.

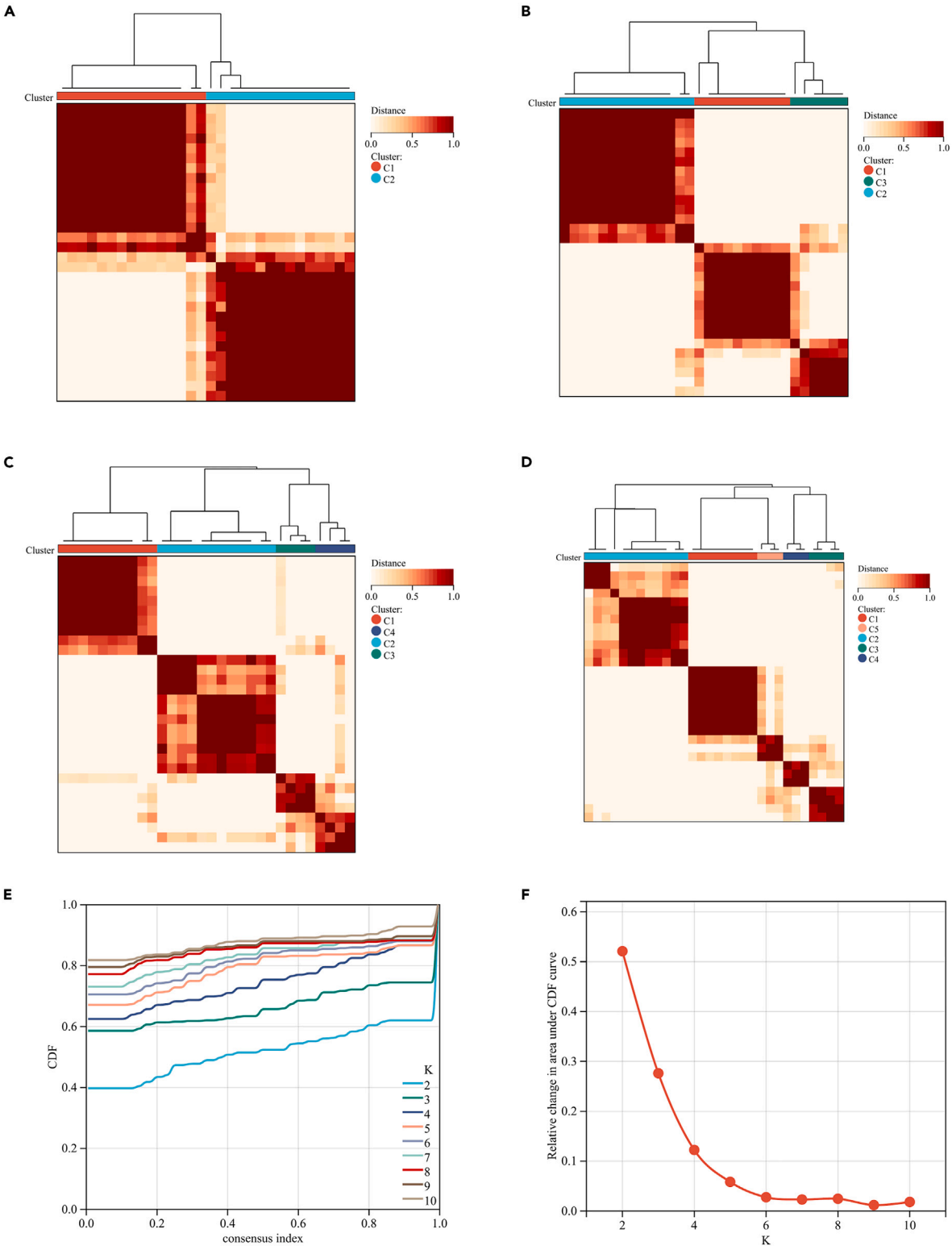

Figure 8. Unsupervised clustering of DFU samples

(A–D) Heatmap of co-occurrence matrix for DFU samples.

(E) Representative cumulative distribution function (CDF).

(F) Relative change in area under CDF curve.

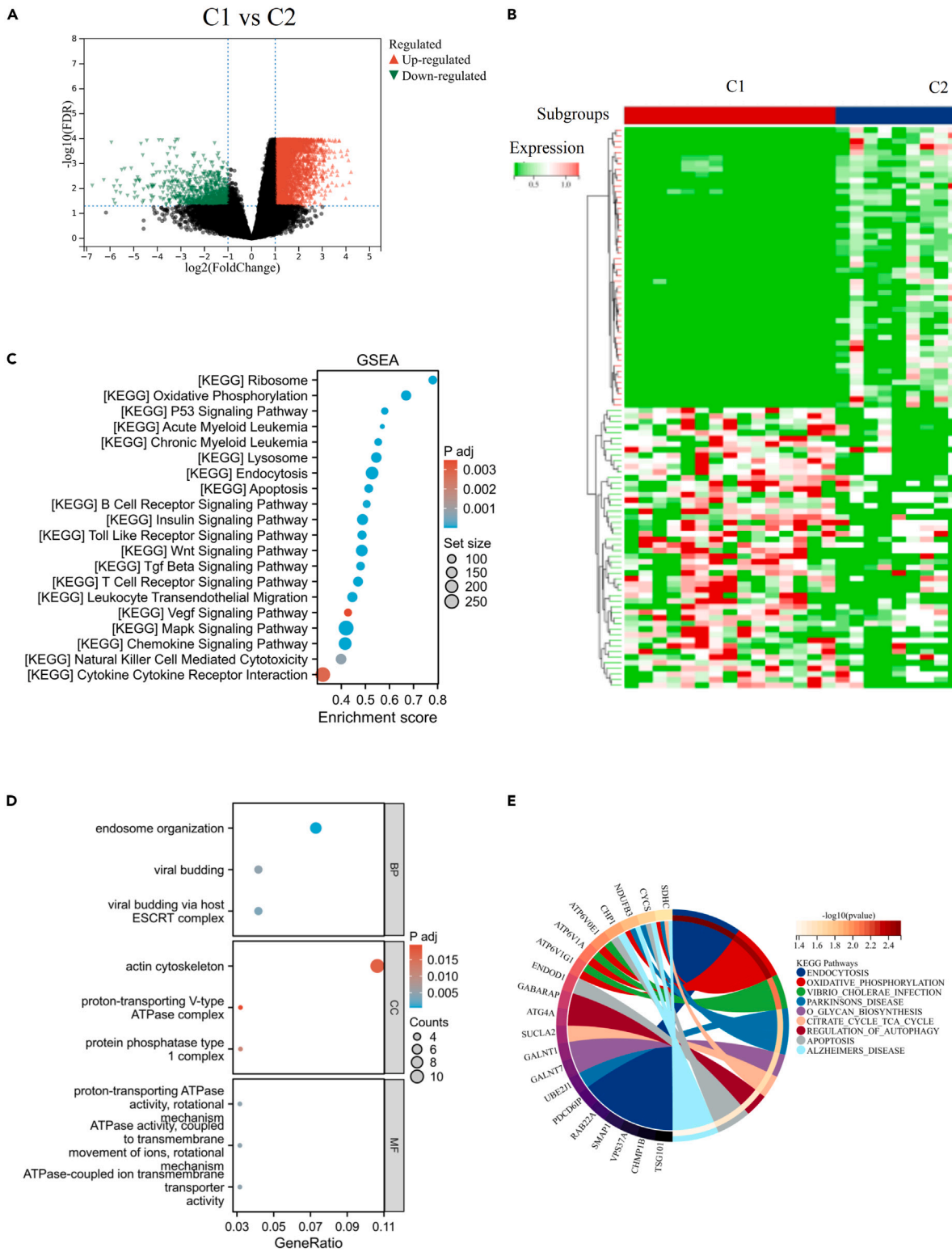

Figure 9. Differential expression and enrichment analysis between the two subgroups

Volcano plot (A) and heatmap (B) of DEGs between C1 and C2 subgroups.

(C) Bubble diagram presented the results of GSEA. The results of GO (D) and KEGG (E) enrichment analysis of DEGs.

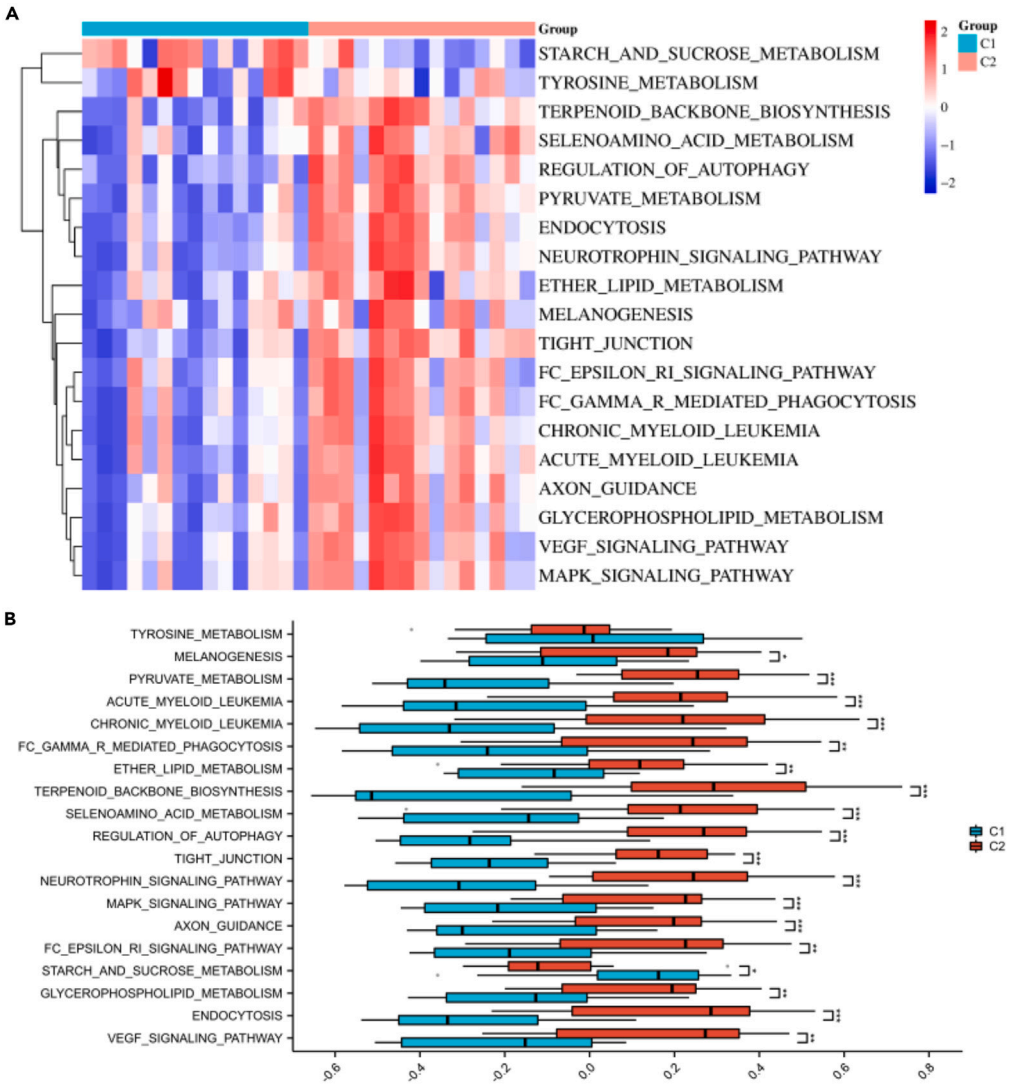

Figure 10. GSVA between the two subtypes

Heatmap (A) and histogram (B) presented the results of GSVA. Statistical comparison between the two subgroups was performed using the Wilcoxon rank-sum test. *p < 0.05, **p < 0.01, ***p < 0.001.

Limitations of the study

Nonetheless, the study has certain constraints. The relatively small sample size may have introduced some degree of bias into the analytical outcomes. To enhance the credibility of the signature genes, we plan to expand our sample collection for more comprehensive evaluation. Additionally, cell experiments ought to be conducted to investigate the molecular processes associated with the signature genes implicated in DFU.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
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  - Materials availability
  - Data and code availability

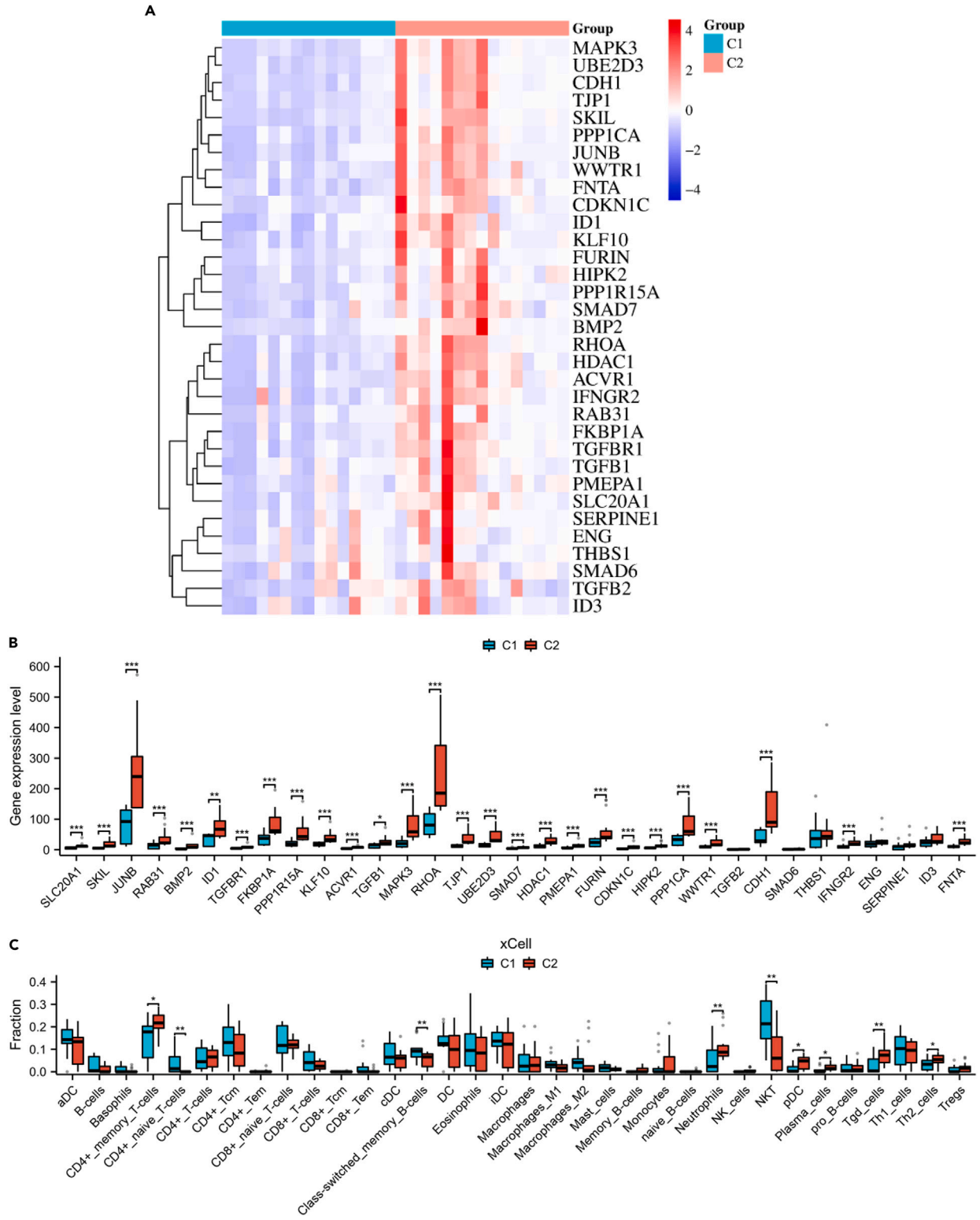

Figure 11. Differences between the two TGF subtypes in TRGs expression levels and immune infiltration

Heatmap (A) and boxplot (B) of expression levels of 33 TRGs in two subgroups.

(C) Boxplot of enrichment scores of 34 immune cells in two subgroups. Statistical comparison between the two subgroups was performed using the Wilcoxon rank-sum test. *p < 0.05, **p < 0.01, ***p < 0.001.

- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Quantitative real-time polymerase chain reaction (qRT-PCR) analysis
  - Ethics approval
- METHOD DETAILS
  - Collection and processing of microarray data
  - Comparison of TGF-β signaling pathway (TGF) score between DFU and NDFU groups
  - Identification of DETRGs in DFU
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  - Identifying diagnostic markers and constructing a nomogram model
  - Immune cell infiltration profile
  - Single-gene gene set enrichment analysis (GSEA)
  - Recognition of distinct TGF subtypes through unsupervised clustering
  - Gene Set Variation Analysis (GSVA)
  - Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109094.

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AUTHOR CONTRIBUTIONS

G.D. and J.C. wrote the manuscript. X.Z. analyzed the data and produced the figures. Z.Z. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
transcriptome data	GEO (https://www.ncbi.nlm.nih.gov/geo/)	GEO: GSE134431
transcriptome data	GEO (https://www.ncbi.nlm.nih.gov/geo/)	GEO: GSE143735
Biological samples		
Foot skin specimens from DFU and NDFU patients	Chinese Academy of Sciences Sichuan Translational Medicine Research Hospital	N/A
Software and algorithms		
sva (R package)	https://bioconductor.org/packages/3.4/bioc/html/sva.html	N/A
Limma (R package)	https://bioconductor.org/packages/3.0/bioc/html/limma.html	N/A
ggplot2 (R package)	https://bioconductor.org/help/search/index.html?search-bar=ggplot2/	N/A
heatmap (R package)	https://bioconductor.org/help/search/index.html?search-bar=heatmap/	N/A
clusterProfiler (R package)	https://bioconductor.org/help/search/index.html?search-bar=clusterProfiler/	N/A
STRING database	https://string-db.org/	N/A
Cytoscape software	http://www.cytoscape.org/	N/A
enrichplot (R package)	https://bioconductor.org/help/search/index.html?search-bar=enrichplot/	N/A
ConsensusClusterPlus (R package)	https://bioconductor.org/help/search/index.html?search-bar=ConsensusClusterPlus/	N/A
GSVA (R package)	https://bioconductor.org/help/search/index.html?search-bar=GSVA/	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Zongdong Zhu, email: zhuzongdong30006@163.com.

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data: This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.
- Code: This study does not report original code. All codes were used in this study in alignment with recommendations made by authors of R packages in their respective user's guide, which can be accessed at https://bioconductor.org.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Foot skin specimens from 8 DFU and 8 NDFU patients were collected from the Chinese Academy of Sciences Sichuan Translational Medicine Research Hospital. The study obtained written informed consent from all participants and was approved by the Ethics Committee of Chinese Academy of Sciences Sichuan Translational Medicine Research Hospital. The skin tissue's total RNA was extracted with the TRIzol reagent (Invitrogen) as per the manufacturer's instructions. The cDNA Synthesis Kit (Invitrogen) was used for complementary DNA synthesis of the total RNA. qRT-PCR was performed on a CFX96™ real-time PCR system (Bio-Rad Laboratories, Inc.). The $2^{-\Delta\Delta Ct}$ method was used to assess gene expression relative to GAPDH expression. Primers used were listed in Table S2.

Ethics approval

This study was approved by the Ethics Committee of Chinese Academy of Sciences Sichuan Translational Medicine Research Hospital.

METHOD DETAILS

Collection and processing of microarray data

Two datasets, GSE134431 and GSE143735, were obtained from the Gene Expression Omnibus (GEO) database. GSE134431 includes 8 non-diabetic foot ulcer (NDFU) samples and 13 DFU samples, while GSE143735 contains 9 DFU samples. The 8 NDFU are diabetic foot patients who did not develop foot ulcers, not healthy controls. Both datasets were sequenced on different platforms, GPL18573 for GSE134431 and GPL11154 for GSE143735, using human samples (Table 1). The two datasets were merged using the Combat function from the 'sva' R package (v.3.22.0).[46] This resulted in a total of 8 NDFU samples and 22 DFU samples.

Comparison of TGF-β signaling pathway (TGF) score between DFU and NDFU groups

The TGF score was assessed through the Gene Set Variation Analysis (GSVA) method, using the subset 'HALLMARK_TGF_BETA_SIGNALING' from the Molecular Signatures Database. The TGF score matrix was obtained, and result was visualized by a violin plot.

Identification of DETRGs in DFU

65 TRGs were obtained from two TGF-related gene sets (BIOCARTA_TGFB_PATHWAY and HALLMARK_TGF_BETA_SIGNALING) (Table S1). To compare the overall expression of these genes between DFU and NDFU, the 'Limma' package (v.3.22.7) was used, and the results were visualized using the 'ggplot2' (v.3.3.6) and 'heatmap' (v.2.13.1) packages.

Protein-protein interaction network (PPI) and functional enrichment analysis

We conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and Gene Ontology (GO) biological function using the R package 'clusterProfiler'. A p.adjust value of less than 0.05 was considered statistically significant. The PPI network was constructed using the STRING database (https://string-db.org/) and visualized through Cytoscape software (v.3.8.2). The identification of core genes was conducted through the cytoHubba plug-in, utilizing 11 algorithms.[47]

Identifying diagnostic markers and constructing a nomogram model

Logistic regression analysis was employed to identify significant genes linked with DFU, from the PPI. The diagnostic model's accuracy was assessed using receiver operating characteristic (ROC) curve analysis. In recent years, the nomogram has gained significant popularity as a predictive tool in the field of oncology.[48–50] To further investigate the role of important TRGs in DFU diagnosis, we utilized the 'rms' (v.6.4.0) package to develop a nomogram model. The model's accuracy was evaluated using calibration curves and decision curve analysis.[51]

Immune cell infiltration profile

Our study evaluated the enrichment scores of 34 subtypes of immune cells using xCell, as previously reported.[52] The abundance of immune cells was estimated using overall marker genes, and the proportion of each subtype was shown as an enrichment score. The differences in immune cell proportions between different groups were evaluated using the Wilcoxon rank sum test. In addition, The 'ggplot2' (v.3.3.6) package was used to analyse and visualise the correlation between the immune cells and the signature genes in a lollipop plot.

Single-gene gene set enrichment analysis (GSEA)

GSEA is commonly employed for the examination and interpretation of concerted alterations at the pathway level in transcriptomic studies.[53] We employed the 'enrichplot' (v.1.22.0) and 'clusterProfiler' (v.4.10.0) packages to categorize samples into high and low expression groups by utilizing the median values of gene expression levels. To explore the molecular mechanisms of genes based on phenotypic grouping, we obtained the subset 'c2.cp.kegg.v7.4.symbols.gmt' from the Molecular Signatures Database.

Recognition of distinct TGF subtypes through unsupervised clustering

The study utilized the ConsensusClusterPlus (v.1.66.0) package to perform unsupervised cluster analysis based on the expression profile of 33 DETRGs.[54] This allowed for the classification of diabetic foot patients into different subgroups and the determination of the optimal number of clusters.

Gene Set Variation Analysis (GSVA)

GSVA enhances the ability to discern nuanced variations in pathway activity across a sample population.[55] The 'c5.go.symbols' subsets from the MSigDB database were obtained to investigate changes in biological pathways. The 'Limma' (v.3.58.1) and 'GSVA' (v.1.50.0) packages

were utilized to analyze the differences in pathways and biological functions among different subtypes. The results are presented as a box plot. Statistical comparison between the two subgroups was performed using the Wilcoxon rank-sum test. A significance level of $P < 0.05$ was considered statistically significant.

Statistical analysis

Statistical analyses were conducted using R software. The Spearman method was used for correlation analysis, while the Wilcoxon rank sum test was employed to compare differences between two groups. A p value of less than 0.05 was considered statistically significant.