Neutrophil Extracellular Trap-Related Genes and Immune Infiltration in Diabetic Foot Ulcers by Bioinformatics Analysis and Experimental Verification

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Abstract: The study aims to investigate the correlation between diabetic foot ulcer (DFU) and neutrophil extracellular traps (NETs), as well as to identify potential biomarkers. Gene microarray data from the Gene Expression Omnibus (GEO) was employed in this research. The GSE134431 and GSE80178 datasets were obtained for bioinformatics analysis to identify differentially expressed genes, which were then cross-referenced with a curated database of genes associated with neutrophil extracellular traps (NETs). Next, the specificity of these potential genes for the disease was determined via Receiver Operating Characteristic (ROC) analysis. Subsequently, the protein-protein interaction (PPI) networks and the Least Absolute Shrinkage and Selection Operator (Lasso) regression were used to identify potential key genes. Additionally, the MCPcounter algorithm was utilized to evaluate the immune infiltration and analyze the relationship between core genes and immune cell infiltration. Furthermore, the levels of expression of these candidate genes were verified by RT-qPCR. Four core genes (CXCL8, S100A12, CXCL12, S100A9) were identified through ROC and Lasso regression analyses. Moreover, individuals suffering from diabetic foot disease exhibited decreased expression levels of T cells, CD8 T cells, cytotoxic lymphocytes, NK cells, fibroblasts, and myeloid dendritic cells; In contrast, monocytes and neutrophils exhibited elevated expression levels. The expression levels of CXCL12 and CXCL8 were positively linked to a wide range of immune and endothelial cells, whereas S100A12 and S100A9 showed distinct correlation patterns with specific immune cell types. qPCR analysis showed increased expression of CXCL8 and CXCL12 as the levels of glucose increased in vitro experimental hyperglycemia. In conclusion, the intricate interplay among genes and immune cell types suggests that DFU and NETs hold potential as valuable biomarkers for the diagnosis and treatment of these conditions, thereby enhancing their clinical meanings.

***Keywords*:** diabetic foot ulcer, neutrophil extracellular traps, bioinformatics, immune infiltration, lasso regression

1. Introduction

Diabetic foot ulcer (DFU) represents a prevalent and challenging complication arising from diabetes mellitus (DM), constituting a primary cause of disability and mortality among people with diabetes. It is estimated that approximately 15% to 25% of individuals with diabetes may develop a DFU during their lifetimes[1]. Given the escalating global incidence of diabetes, there is an anticipated surge in the number of patients affected by DFU. Furthermore, it was also found that patients with DFU face a higher mortality risk which is 2.5 times in comparison to those without foot wounds[2]. The problem and features encompass prolonged treatment, multiple hospital admissions resulting from persistent infection, gangrene, and a high recurrence rate, which significantly impact patients' quality of life, psychological adjustment, and economic status[1-2]. Recently, this issue has gained global attention as a significant social concern. Current clinical practices to manage diabetic foot ulcers(DFU) primarily focus on glycemic control and infection management alongside necrotic tissue debridement and tibial transverse transport[3]. Despite recent advancements in DFU treatment approaches, their intricate pathophysiological mechanisms remain a crucial factor contributing to treatment failures. Thus, it is crucial to gain a thorough comprehension of the pathophysiological mechanisms of DFU to facilitate the development of novel therapeutic strategies.

Neutrophils, as the most numerous innate immune cells in the body, have a pivotal role in the inflammatory stage of wound healing by being the first leukocytes recruited to the site of inflammation[4]. In 2004, Brinkmann et al. described a novel form of cell death in neutrophils, known as NETosis, wherein activated neutrophils release an identifiable extracellular web-like structure. This structure, known as neutrophil extracellular traps (NETs), consists of a DNA scaffold embedded with

histones, neutrophil elastase (NE), myeloperoxidase (MPO), and antimicrobial peptide (AMP) components[5]. However, recent studies have highlighted a significant connection between the excessive production of NETs in individuals with diabetes and the persistent non-healing characteristic of DFU[6]. Elevated blood glucose levels and the accumulation of metabolites, particularly in foot ulcers, lead to heightened generation of NETs. This triggers a substantial release of pro-inflammatory factors in the wound site resulting in immune cell infiltration and an exaggerated inflammatory response that disrupts the typical process of wound healing[7]. Therefore, it is imperative to thoroughly investigate the correlation between NETs and DFU to comprehend the pathophysiological basis of DFU and develop targeted therapeutic approaches.

In our research, gene set enrichment analysis (GSEA) on raw data from the GEO database were conducted to determined key modules linked to NETs. Subsequent ROC screening was performed for potential target genes identified at the intersection with NETs genes. The diagnostic significance of these genes was assessed exerting the PPI (Protein-Protein Interaction) network and Lasso regression analyses. Immune Infiltration analysis was accomplished on key genes, followed by an examination of immune cell infiltration patterns using MCPCounter. Additionally, correlations between key genes and immune cells were explored. Finally, this work may contribute to identifying potential biomarkers for DFU therapy and enhancing our understanding of DFU pathogenesis.

2. Methods

2.1 Data Download and Preparation

The GEOquery [8]package was employed to retrieve data from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) regarding GSE134431[9] and GSE80178[10] for analysis in this study. Specifically, the dataset GSE134431, based on the platform GPL18573, consisted of samples obtained from 13 patients suffering diabetic foot ulcer and 8 subjects having diabetic foot skin. To identify genes associated with NETs, a systematic literature review was conducted using "Neutrophil extracellular traps" as the search term.

2.2 Identification of DEGs

Differential expression analysis was conducted on samples collected from patients suffering DFU and those with diabetic normal foot skin in this study. Data normalization was performed using the ‘normalize between arrays’ function of the R language “limma” package[11]. Additionally, the probes were precisely noted based on the GPL platform annotation file, excluding those mapping to multiple genes. In cases where genes had multiple probes, we selected the probe with the strongest signal for further analysis. Principal component analysis (PCA) plots demonstrated clustering among samples. To pinpoint differentially expressed genes (DEGs) between the two groups, we employed the "limma" package, setting the criteria at $|LogFC|>2$ and $P.adj<0.05$. Finally, volcano plots were utilized to illustrate the outcomes of the differential analysis.

2.3 Functional Enrichment Analysis of DEGs

The gene set enrichment analysis (GSEA) was performed using the “clusterProfiler” software package [12]. Gene sets were considered significantly enriched if they met the criteria of having an adjusted p-value (P.adj) below 0.05 and a false discovery rate (FDR, i.e., q-value) under 0.25. To explore the similarity between enriched terms, we calculated the Jaccard similarity index (JC) and classified the enriched terms through hierarchical clustering analysis (hclust). Finally, the clustering outcomes were then visually displayed using the “ggplot2” package.

2.4 Receiver Operating Characteristics (ROC) Analysis of Intersection Genes

The differentially expressed genes identified were intersected with NETs-related genes to pinpoint potential target genes. Subsequently, ROC analysis was conducted on these potential targets using the “PROC” package. Furthermore, validation was performed within the GSE80178 dataset.

2.5 PPI Network and Lasso Analysis

The PPI network analysis was performed through the Search Tool for the Retrieval of Interacting Genes (STRING) database, setting the confidence threshold at 0.7 and exclusion of genes with limited connectivity. Subsequently, LASSO regression analysis was performed for diagnostic purposes.

2.6 Analysis of Immune Infiltration

Immune infiltration analysis in diabetic foot ulcer versus normal foot skin was performed through the MCPcounter package[13] in R, which enables the quantification of the absolute abundance of eight types of immune cells and two types of stromal cells from transcriptomic data. The permutation test iterations were set to 100 for the analysis. Infiltration levels

were visualized using the “ggPlot” package. Additionally, Spearman's statistical method was exerted to assess relationship between key genes and immune cell abundance, with visualization through the “ggPlot2” package.

2.7 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco's Modified Eagle’s Medium (DMEM, Gibco, USA), enriched with 10% fetal bovine serum (FBS, Gibco, USA) and 100μg/ml penicillin-streptomycin.

2.8 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) Analysis of Hub Genes

Following reaching confluency, the HUVEC were inoculated into 6 cm culture dishes for 24 hours. Then, complete medium containing different glucose concentrations (25mM, 50mM, 100mM) was added and incubated for 2 days for the further experiment. The procedure of total RNA and cDNA was doned following the instruments of test kits (Fastagen RNA fast200 kit and BeyoRT™ III First Strand cDNA Synthesis Kit with gDNA EZeraser). Primers for the target genes were designed using NCBI Primer-Blast software. Primer sequences were showed in Table 1.

Table 1. The primer sequences of key genes.

Gene Name	Primer sequences (5’ to 3’)	
CXCL-8	F	TTTTGCCAAGGAGTGCTAAAGA
	R	AACCCTCTGCACCCAGTTTTC
CXCL-9	F	ATGGCCCTGTGCCTTAGTAGT
	R	AGCTTTGCATTCATGGTCTTGA
β-actin	F	TGACGTGGACATCCGCAAAG
	R	CTGGAAGGTGGACAGCGAGG

3. Results

3.1 Identification of DEGs in DFU

The GSE134431 dataset was analyzed using R language to identify differential genes between patients with diabetic foot ulcers (DFU) and normal subjects (Figure 1A). A total of 2082 differential genes were identified grounded on the screening condition of |LogFC|>2 & P.adj<0.05. Among these, 241 genes showed up-regulated expression while 1841 genes exhibited down-regulated expression (Figure 1B). To identify key genes involved NETs formation in the patients with DFU, the differentially expressed genes from GSE134431 were intersected with NETs, resulting in the identification of 29 common differential genes (Figure 1C).

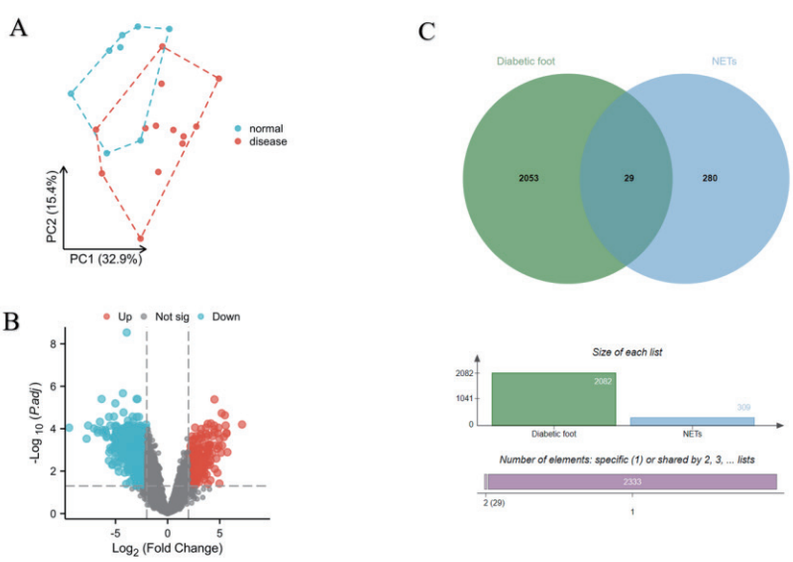

Figure 1. The Data Analysis of GSE134431. (A)PCA plot of GSE134431. (B)Volcano maps display differentially expressed genes found in DFU patients in GSE134431. (C)Venn diagram representing NETs in DFU patients in GSE134431.

3.2 GESA Enrichment Analyses of DEGs

Enrichment analysis was performed on the genes, identified as differentially expressed, revealing significant enrichment in the angiogenesis signaling pathway through clustering of similar outcomes (Figure 2A). Angiogenesis, the formation of new blood and lymphatic vessels from existing vasculature, is crucial in embryonic development, wound healing, and various physiological processes. Additionally, enrichment was observed in pathways related to burn wound healing and cytokine signaling within the immune system, indicating that these differentially expressed genes are essential for the wound healing and immune response. The analysis also uncovered a notable enrichment in the pathway of neutrophil extracellular traps (Figure 2B), suggesting their significant involvement in these biological processes.

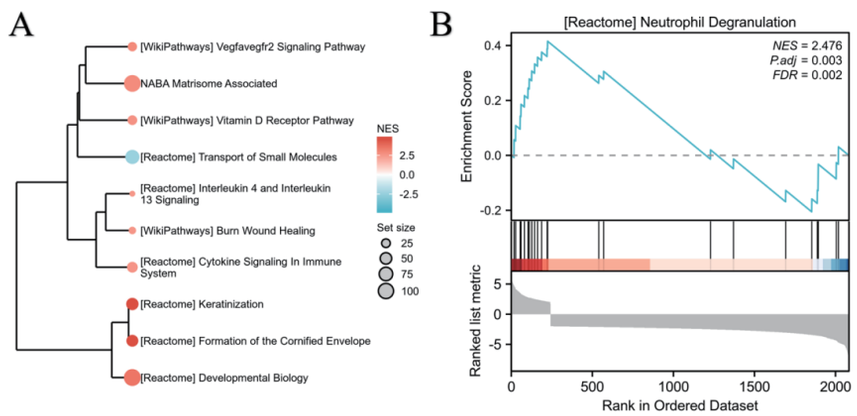

Figure 2. Gene Set Enrichment Analysis. (A) Visualization of significantly enriched pathways. (B)The pathway of neutrophil extracellular traps.

3.3 ROC Diagnostic Analysis of DEGs

The intersecting genes identified previously were further validated using ROC regression analysis. By examining ROC curves, genes with superior discriminatory abilities, specifically those with an AUC exceeding 0.7, were selected (Figure 3A-C). Significant disparities in gene expression have been noted in tissue from diabetic foot ulcer and normal foot skin. Subsequent analysis indicated higher expression levels of genes such as ANXA1, S100A12, S100A9, SLPI, S100A8, CXCL8, CXCR1, MMP1, IL36RN, and FBW2 in diabetic foot ulcer samples, while genes like CXCL12, C3, and KI7 exhibited lower expression levels in diabetic foot ulcer (Figure 3D). Validation conducted on the GSEGSE80178 dataset confirmed the diagnostic value of the differentially expressed genes (Figure 3E, F).

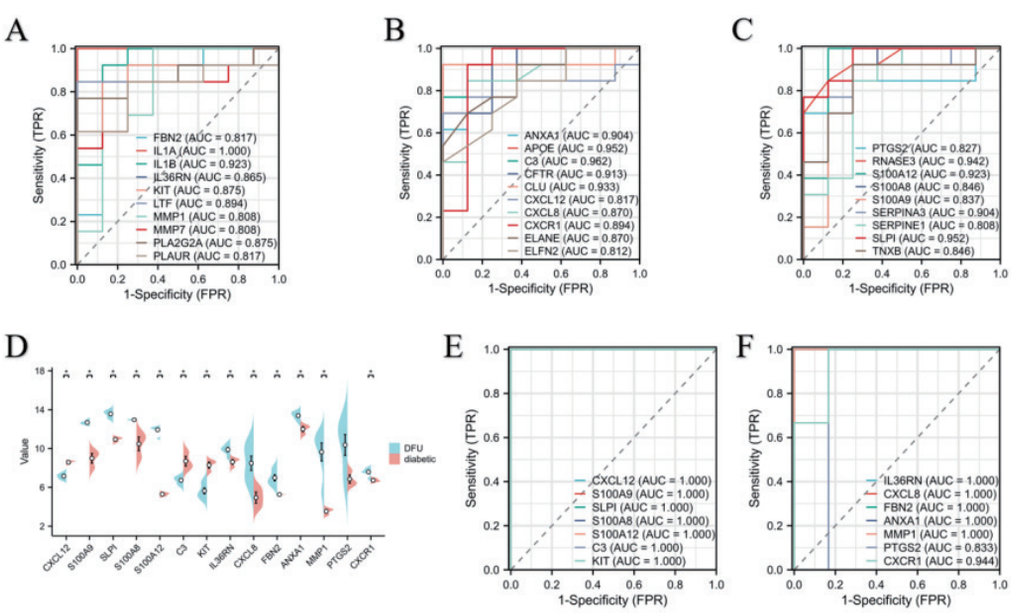

Figure 3. ROC Analysis and Differential Analysis. (A-C) ROC diagnostic analysis of intersection genes based on GSE134431. (D) Differential expression of intersection genes. (E-F) ROC diagnostic analysis of intersection genes based on GSE80178.

3.4 Lasso Diagnostic Analysis

The interactions among the 13 genes were analyzed using the STRING database. IL36RN, FBN2, and SLPI were excluded because they were not directly linked to other genes (Figure 4A). Variable selection was achieved through Lasso regression, which penalizes the magnitude of the regression coefficients. The parameter of the penalty strength is represented by λ (lambda). The study findings indicate that the minimum mean square error is associated with a λmin value of 0.084521, while a λ1se value of 0.28328 is associated with the maximum value within one standard error from the minimum mean square error. As λ increases, the regression coefficients in the model decrease, and some coefficients may be compressed to zero, indicating that certain genes are excluded from the model and lack diagnostic value. When λ is set to λmin=0.28328, the model retains four coefficients, indicating that four genes (CXCL8 and CXCL12) may be relevant to the diagnosis of diabetic foot disease (Figure 4B, C).

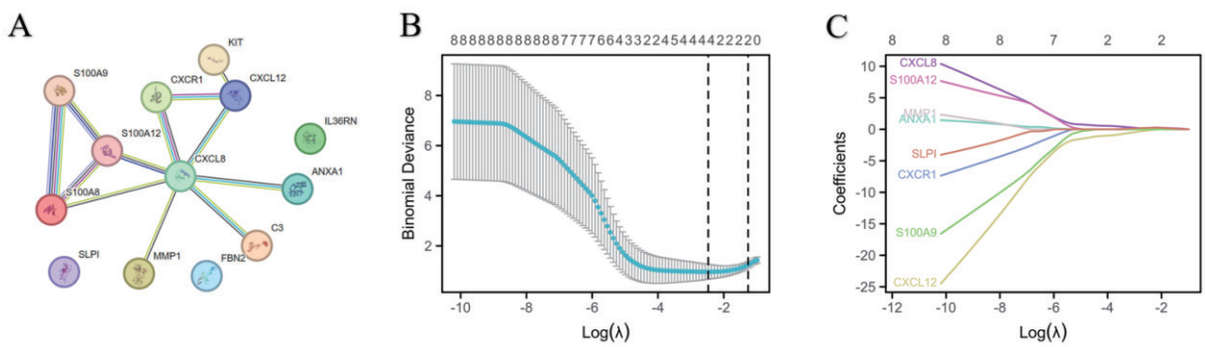

Figure 4. PPI Network Analysis and LASSO Regression. (A) PPI network interactions. (B) Coefficient selection through Lasso logistic regression for genes with nodes. (C)Variable trajectories in Lasso logistic regression.

3.5 Immune Infiltration Analysis

The analysis of immune infiltration was performed using MCPCounter to investigate alterations in immune cell populations among patients with diabetic foot disease. The results of this research revealed that a significant decrease in the expression levels of T cells, CD8 T cells, cytotoxic lymphocytes, NK cells, fibroblasts, and myeloid dendritic cells in patients with diabetic foot disease versus the control group. Conversely, there was a notable increase in the levels of expression of monocytes (Monocytic_kineage) and neutrophils in individuals with diabetic foot disease (Figure 5).

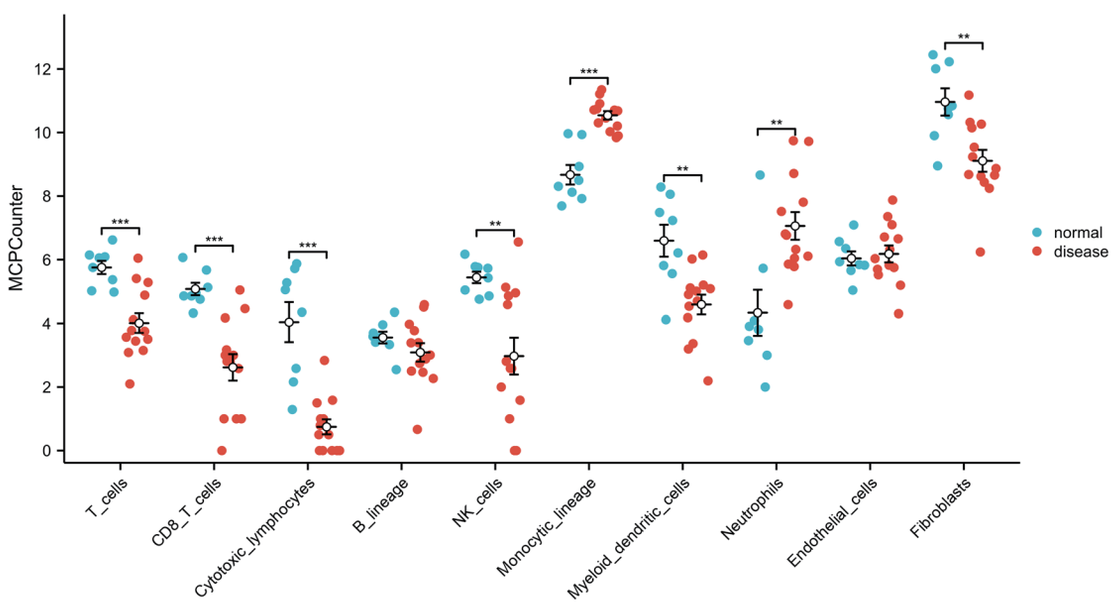

Figure 5. The Analysis of Immune Cell Infiltration Using the MCPcounter Algorithm.

3.6 Correlation Analysis of Hub Genes with Immune Infiltration

The screened Hub genes underwent correlation heatmap analysis with immune cells. The analysis revealed that in the diabetic foot disease group, CXCL12 exhibited positive correlations with T-cells (R=0.753), cytotoxic lymphocytes (R=0.762), B-cells (R=0.588), NK cells (R=0.964), neutrophils (R=0.731), endothelial cells (R=0.813), and fibroblasts (R=0.907). CXCL8 showed positive correlations with B lymphocytes (R=0.588), NK cells (R=0.65), neutrophils (R=0.973), endothelial cells (R=0.593), and fibroblasts (R=0.632). Within the diabetic group, CXCL12 correlated positively with T-cells (R=0.714) and B-cells (R=0.81); S100A12 with monocytes (R=0.783); and S100A9 negatively with cytotoxic lymphocytes (R=-0.81) and fibroblasts (R=-0.857), but positively with monocytes (R=0.738). In the heatmap, varying colors (red or blue) indicate the strength of correlation, with red for positive and blue for negative correlations. The intensity of the color signifies the degree of correlation (Figure 6).

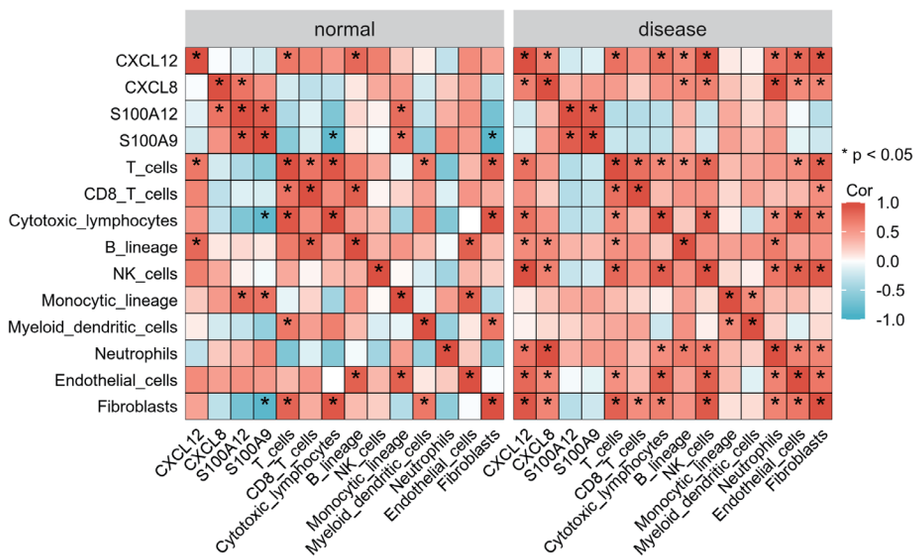

Figure 6. Correlation analysis among expression of core genes and immune cells.

3.7 Quantitative analysis of key genes

RT-qPCR analysis revealed significant variability in the RNA expression levels of CXCL-8 (Figure 7A) and CXCL-12 (Figure 7B), suggesting a positive correlation between their expression and elevated glucose concentrations, which is in agreement with the results of previous bioinformatic analysis (Figure 4B-C). The findings validate the trend of increasing expression levels of CXCL-8 and CXCL-12 genes under conditions of increasing glucose concentration.

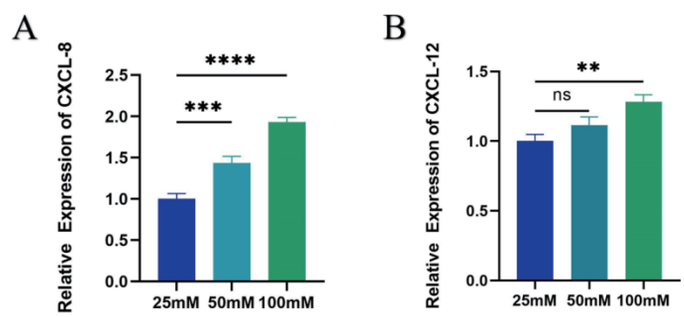

Figure 7. Expression of key diagnostic genes in HUVEC cells under varying glucose conditions validated by RT-qPCR (n=3). (A)Expression levels of CXCL-8. (B)Expression levels of CXCL-12.

4. Discussion

Neutrophil extracellular traps (NETs) are strongly correlated with disease progression in patients suffering diabetic foot disease. Diabetic patients exhibit significantly elevated blood levels of released NETs products, which are correlated

with complications including delayed-healing wound, diabetic retinopathy, and atherosclerosis[14]. The formation of NETs depends entirely on glucose and partially on glycolysis[15]. A hyperglycemic environment may promote the generation of NETs, which can affect the development of diabetic foot disease. NETs have a dual role in diabetic foot disease, causing tissue damage and inflammation while also trapping and destroying pathogens. However, excessive or persistent NETs may lead to delayed healing of diabetic foot ulcer wounds[16]. Therefore, it is imperative to investigate the association of diabetic foot disease with the formation of NETs. This study conducted a comprehensive bioinformatics analysis to examine the key genes, functions, as well as immune cells associated with NETs in these patients.

The analysis of the GSE134431 dataset in this study revealed that the genes expressing differentially were significantly enriched in signaling pathways, for example angiogenic signaling pathways, burn wound healing, cytokine signaling among the immune system, and NETs. It is critical to regulate angiogenesis for endothelial cell (EC) homeostasis. Endothelial-to-mesenchymal transition (EndMT) indicates EC plasticity[17]. Previous research has demonstrated a link between EndMT and reduced levels of angiogenesis in the process of wound healing[18]. Moreover, NETs can induce EndMT by disrupting VE-calmodulin as well as subsequently activating β-catenin signaling[19]. Additionally, diabetes may trigger the immune response in patients. The MCPCounter algorithm was employed to analyze altered immune cell populations in patients with diabetic foot disease. Myeloid dendritic cells play a key role as antigen-presenting cell that activates T cells. In patients with diabetic foot disease, reduced expression of these cells may impair antigen presentation and immune activation processes. Additionally, studies have observed a droplet in the expression of T cells and cytotoxic lymphocytes, particularly CD8 T cells. The reduction in Natural Killer (NK) cells levels compromises their ability to recognize and eliminate virus-infected cells, thereby impairing the immune response and possibly contributing to disease progression. The diminished expression of NK cells also suggests immune dysfunction that may be caused by diabetes. Fibroblasts, the predominant cell type in connective tissue, play a vital role in tissue repair and fibrosis.[20]. The reduced expression of fibroblasts could hinder the healing process in patients suffering from DFU. It has been demonstrated that the fibroblast growth factor (FGF) family, through its specific receptors, can orchestrate various processes including angiogenesis, skin wound-healing, metabolic regulation, and embryonic development[21]. Certain FGF isoforms may influence the diabetic wound healing process by affecting gene expression levels of α-FGF and β-FGF which have been observed to decline during early stages[22]. Notably, α-FGF significantly increases capillary density and promotes fibroblast proliferation within ulcerated tissues while enhancing transforming growth factor-β expression along with proliferating cell nuclear antigen (PCNA), thus improving diabetic ulcer conditions[23].

The study revealed that the patients of DFU exhibited increased levels of expression for monocytes and neutrophils, indicating an immune imbalance that potentially contributes to disease progression through enhanced inflammatory response. Further analysis of neutrophil-associated genes using ROC and Lasso regression identified four diagnostic biomarkers: CXCL8, S100A12, CXCL12, and S100A9. S100A9 and S100A12 belong to the S100 protein family, which upregulates endothelial cell adhesion molecules, activates inflammatory cells, possesses chemotactic properties, and has antimicrobial functions[24]. Neutrophils and monocytes are the main cellular sources expressing S100A9 and S100A12. In this study, there was a significant positive correlation between monocytes and S100A9 as well as S100A12. Although there was a positive correlation with neutrophils as well, it did not reach statistical significance.

It has been observed that multiple cytokine and chemokine genes involving in wound healing exhibit single nucleotide polymorphisms (SNPs). Specifically, SNPs in the CXCL-12 gene have been linked to a high risk of severe foot microbial infections, amputations, and advanced foot ulcer in patients with DM[25]. Further researches have also revealed a connection between abnormal CXCL-12 production, chronic inflammation and reduced angiogenesis and impaired healing[26]. In our study, we discovered that HUVEC interacted under high glucose conditions exhibited increased levels of CXCL-12 expression. In addition, targeting CXCL-8 in hard-to-heal DFU can promote wound healing process with the reducing levels of chronic inflammation. Optimal levels of CXCL-8 are essential for normal wound repair in the stages of proliferation and remodeling while playing a pivotal role in extracellular matrix (ECM) remodeling[27]. Similarly, we also observed elevated levels of CXCL-8 expression in HUVEC under a high glucose culture environment, consistent with previous findings[28]. Manduri Sathvik et al. [29]used the Neutrophil-to-Lymphocyte ratio (NLR) as a powerful indicator to evaluate the healing status with DFU. NLR remains stable and unaffected by physiological or environmental changes which could otherwise impact accurate assessment of diabetic foot ulcer progression. The NLR serves as an enduring marker resistant to physiological or environmental fluctuations unlike other markers including dehydration, exercise or blood sample processing [30]. Numerous studies have demonstrated the role of NLR in systemic inflammation associated with diabetes. [31-32]

5. Conclusion

In summary, this study utilized a bioinformatics approach to explore the immune profile of patients with DFU, particularly focusing on the association with neutrophil extracellular traps. The diagnostic significance of CXCL-8 and CXCL-12 genes was identified, along with their distinct regulatory mechanisms involving monocytes and cytotoxic lymphocytes. Additionally, this study has certain limitations, including the lack of in vivo animal experiments and clinical trials. Further research is required to confirm and validate these findings in future studies.

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