Exploration of the Shared Gene Signatures and Molecular Mechanisms Between Diabetic Foot Ulcer and Diabetic Microvascular Disease

Zhuodong Fu, Junwei Zong, Shouyu Wang

Department of Orthopedic Surgery, the First Affiliated Hospital of Dalian Medical University, Dalian 116011, China.

Abstract: Background: Diabetic foot ulcer (DFU) is a serious complication of diabetes caused by multiple factors. Diabetic microvascular disease has a close linkage with DFU. However, the inter-relational mechanisms between them are still unclear. This article aimed to explore the shared gene signatures and potential molecular mechanisms in DFU and diabetic microvascular disease. Methods: In the GEO database, DFU microarray datasets (GSE80178, GSE68183) and diabetic microvascular disease microarray datasets (GSE43950) were downloaded. After data standardization processing, we used R software to analyze the transcriptome sequencing data of each data set to find the differentially expressed genes (DEGs) of DFU and diabetic microvascular disease. Then obtained the overlapped DEGs in DFU and diabetic microvascular disease database by Jvenn. Finally, the shared DEGs were enriched by pathway enrichment and protein-protein interaction (PPI) analysis, and the hub gene was found by node analysis. Results: Totally, 1007 DEGs were identified in the GSE80178 dataset, 338 DEGs were identified in the GSE68183 dataset, 1154 were identified in the GSE43950 dataset, Venn diagram analyses showed that there were 14 shared DEGs in these datasets. Enrichment analysis shows that the shared DEGs were mainly associated with chronic inflammatory response, leukocyte migration, cellular transition metal ion homeostasis, vascular wound healing, collagen-containing extracellular matrix and Toll-like receptor binding. Involved pathways were mainly enriched in IL-17 signaling, glycosaminoglycan degradation, and calcium signaling. PPI analysis of these shared DEGs shows that S100A9, S100A8, CSTA, ADAP2, CD34 and FGL2 were the hub gene whose plays a pivotal role in DFU.Conclusion: Our work has identified several new DFU candidate genes that can be used as biomarkers or potential therapeutic targets.

Keywords: Diabetic Foot Ulcer; Diabetic Microvascular Disease; Differential Gene Analysis

1. Introduction

Diabetes mellitus (DM) has become one of the most serious public health problems worldwide. According to the latest data, the global prevalence of DM has reached 10.5% in 2021 and this number is expected to reach 12.2% in 2045, approximately 783.2 million people[1].

Poorly controlled diabetes lead to variety of chronic complications, such as microvascular disease, diabetic foot ulcer (DFU) and peripheral neuropathy. DFU is a disease caused by many factors, which is defined as the destruction of skin and its deep tissue far away from the ankle in patients with DM, always complicated with infection and (or) arterial occlusion of the lower extremities, and in severe cases involving muscle and bone tissue[2]. It is one of the most common and serious complications of DM. Microvascular disease are mainly characterized by thickened capillary basement membrane and

microthrombosis. Under the action of multiple factors, the morphological function and metabolic function of microvessels and microblood flow are seriously impaired, which leads to organ and tissue damage. Currently, there are many clinical studies on the association between DFU and diabetic microvascular disease, but mainly limited to the histological and functional levels, fewer at the molecular level.

Using the published gene expression data from the Gene Expression Omnibus (GEO) (http://www. ncbi.nlm.nih.gov/geo/), we identified the shared gene between DFU and diabetic microvascular disease, explored the underlying molecular mechanisms. It may help us to explore new diagnostic markers or therapeutic targets for DFU.

2. Materials and Methods

2.1 Dataset Download

We downloaded the DFU and diabetes-related microvascular complications gene expression profiles in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/ geo) database. The following criteria filter the downloaded dataset: First, the gene expression profiling must include cases and controls. Second, these datasets must provide the processed data or raw data that could be used for reanalyzation. Finally, the GEO dataset numbered GSE80178, GSE68183 and GSE43950 were selected. The GSE80178 and GSE68183 datasets were tested on the platform of Affymetrix Human Gene 2.0 ST Array. The GSE43950 dataset was tested on the Rosetta/Merck Human RSTA Custom Affymetrix 2.0 microarray platform.

2.2 Identification of Shared and Unique Gene Signatures in DFU and Diabete Microvascular Disease

The “limma” package (version3.50.1) in R software (version4.1.2) was used to normalize the gene expression data and identify the differentially expressed genes (DEGs) between the case and control group. The FDR < 0.05, $|logFC| > 0.5$ (GSE80178, GSE43950) and $|logFC| > 1$ (GSE68183) were considered to be threshold values. The “pheatmap” package (version 1.0.12) in R software was used to perform the hierarchical clustering heat maps that reveal the expression patterns of these DEGs. The overlapped DEGs in DFU and diabetes-related microvascular complications database were obtained using Jvenn.

2.3 GO and KEGG Analyses

The biological functions of identified DEGs of interest were assessed using the Database for Annotation, Visualization, and Integrated Discovery version (DAVID) Bioinformatics Resources (v6.8). Briefly, shared DEGs were imported into DAVID, and Gene Ontology (GO) and KEGG enrichment analyses were then conducted. For GO analyses, enriched biological processes (BPs), molecular functions (MFs), and cellular components (CCs) were assessed. The “GOplot” R package was used to visualize the results of these enrichment analyses.Then, DEGs were imported into Search Tool for the Retrieval of Interacting Genes (STRING) to construct the protein-protein interaction (PPI) network. The TSV file of PPI network was imported into Cytoscape 3.7.1. The interactions between enriched KEGG pathways were calculated and visualized by Cytoscape 3.7.1.

2.4 Common miRNAs-Target Genes Network Construction

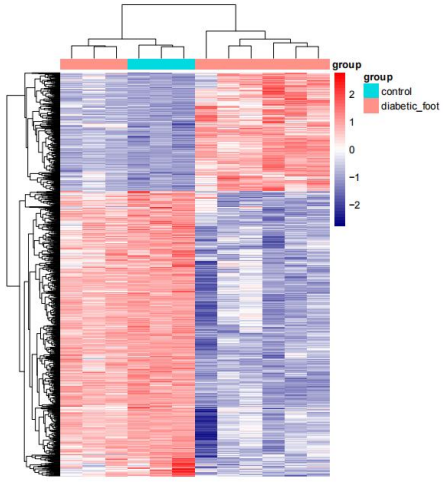
The miRTarbase is an experimentally validated miRNA-target interactions database (http://mirtarbase. mbc.nctu.edu.tw/ php/index.php), which included 4,076 miRNAs and 23,054 target genes supported by experimental evidence (reporter assay, Western blot, microarray, or pSILAC). The intersection of target genes of common miRNAs and shared genes in DFU and

diabete microvascular disease were used to construct the miRNAs–mRNAs regulated network. Cytoscape software was used to visualize the network.

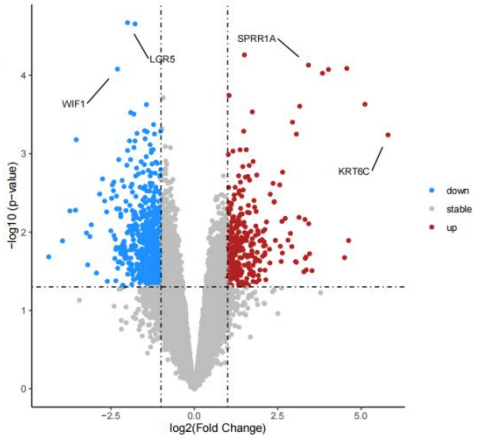
3. Result

3.1 DEG Identification

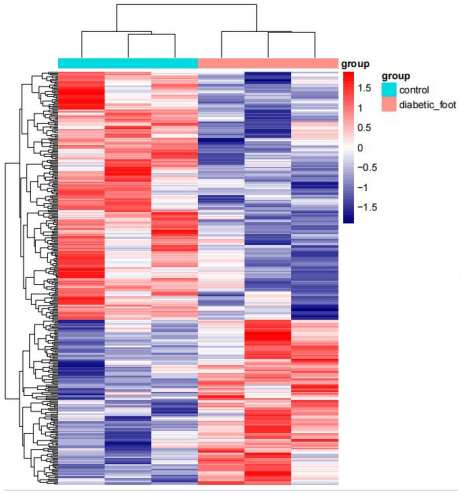
The GSE80178, GSE68183 and GSE43950 gene expression datasets were obtained from the GEO database. The GSE80178 dataset included 9 diabetic foot biopsy samples and 3 biopsy samples from normal individuals, GSE68183 dataset included 3 diabetic foot biopsy samples and 3 biopsy samples from normal individuals, whereas the GSE43950 dataset included 5 diabetic microvascular disease biopsies and 9 biopsies from healthy individuals. In total, 1007 DEGs were identified in the GSE80178 dataset (296 upregulated, 711 downregulated), 338 DEGs were identified in the GSE68183 dataset (135 upregulated, 203 downregulated), while 1154 were identified in the GSE43950 dataset (821 upregulated, 333 downregulated). The volcano plot and the heatmap of all DEGs were visualized respectively (Figure 1). Totally, 14 DEGs were shared between these three datasets, as identified through Venn diagram analyses (Figure 2).



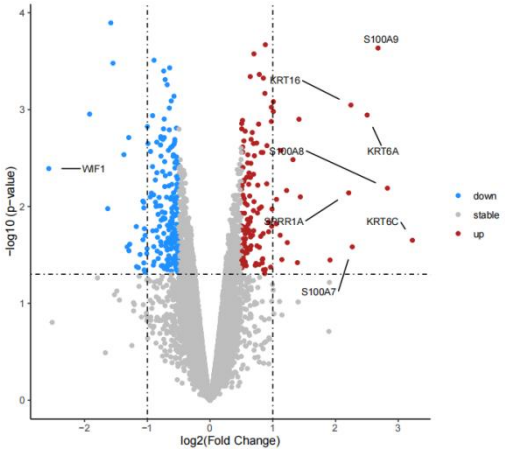
(a)



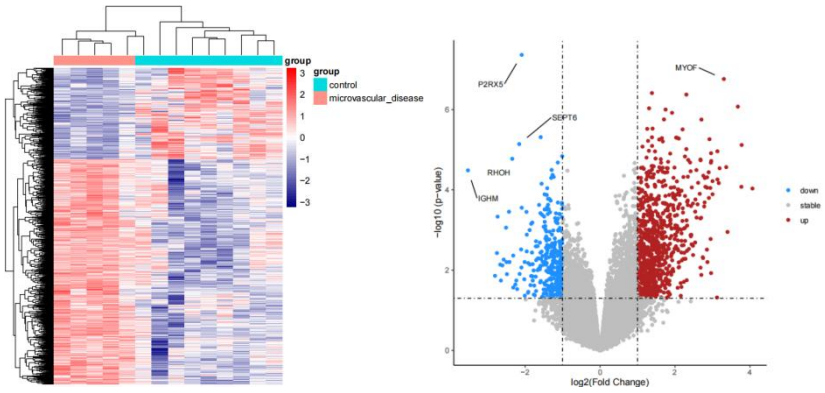
(b)



(c)



(d)



(e) (f)

Figure 1: Detection of differentially expressed genes (DEGs) in the GSE80178, GSE68183 and GSE43950 datasets. (a) An expression heat map of all the DEGs in the GSE80178 dataset, as determined based upon P values. (b) A volcano plot corresponding to the GSE80178 dataset. (c) An expression heat map of all the DEGs in the GSE68183 dataset, as determined based upon P values. (d) A volcano plot corresponding to the GSE68183 dataset. (e) An expression heat map of all the DEGs in the GSE43950 dataset, as determined based upon P values. (f) A volcano plot corresponding to the GSE43950 dataset.

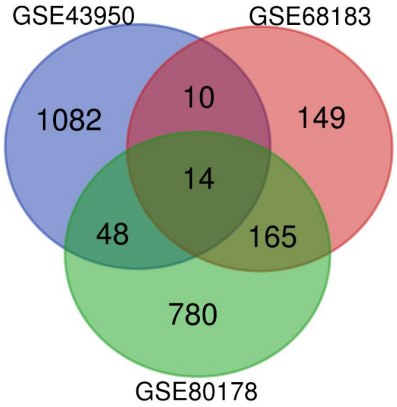
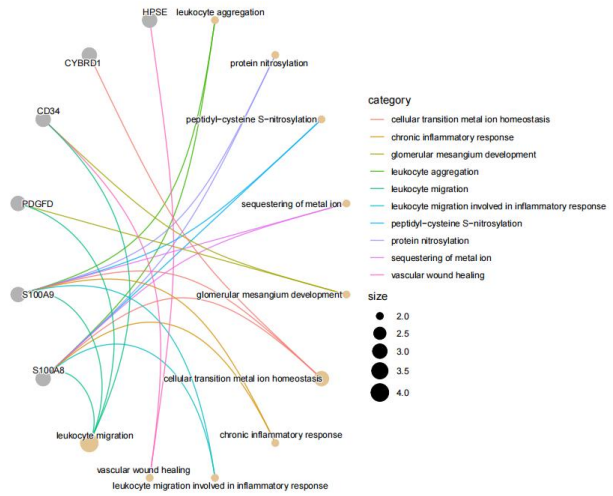


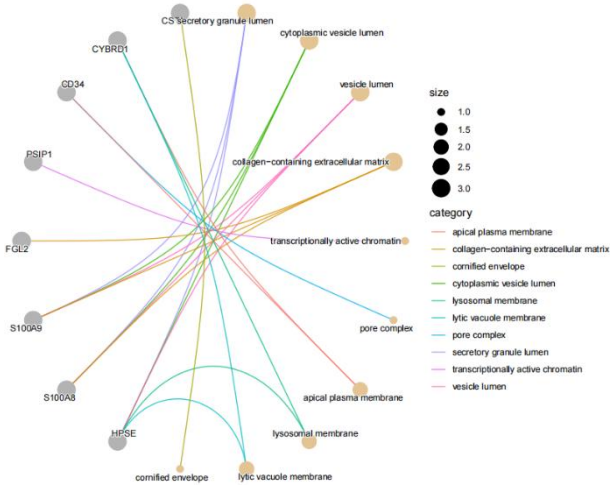
Figure 2: Identification of shared DEGs in GSE80178, GSE68183 and GSE43950.

3.2 GO and KEGG Analyses

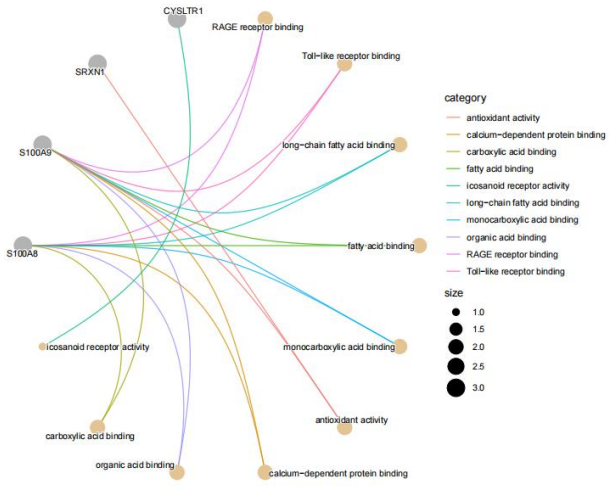
The biological functions and pathways analyses were conducted using R package clusterProfifiler. The three GO categories (BP, CC, MF) of the shared DEGs were enriched respectively (Figure 3). The shared DEGs were mainly associated with chronic inflammatory response, leukocyte migration, cellular transition metal ion homeostasis, vascular wound healing, collagen-containing extracellular matrix and Toll-like receptor binding. As shown in Figure 4, the involved pathways were mainly enriched in IL-17 signaling (hsa04657), glycosaminoglycan degradation (hsa00531), and calcium signaling (hsa04020).



(a)



(b)



(c)

Figure 3: The top 10 terms of GO categories. (a) Biological process (BP). (b) Cellular component (CC). (c) Molecular function (MF).

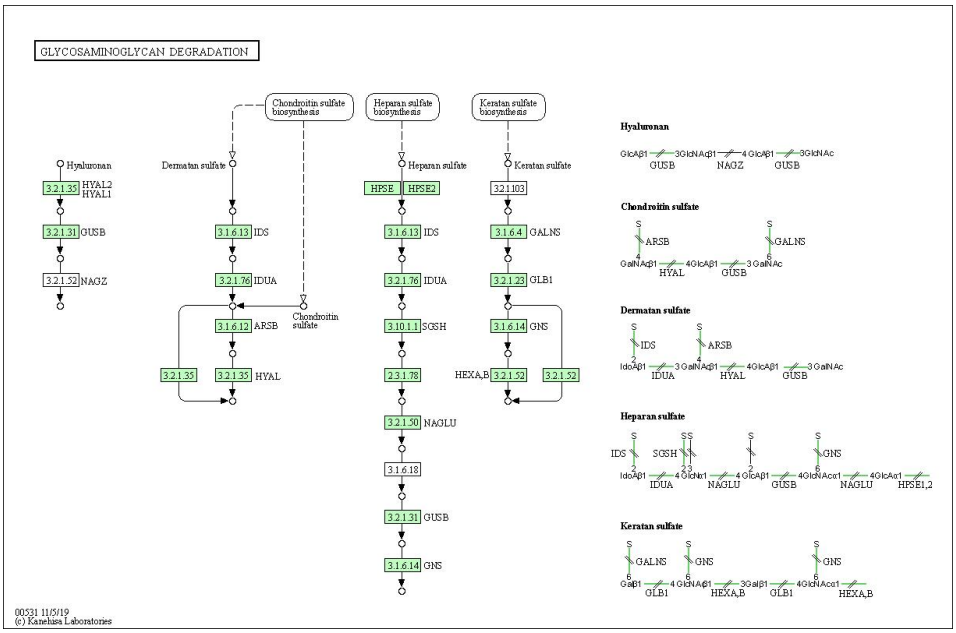


Figure 4: KEGG pathway analysis of the shared DEGs.

3.3 PPI Network Construction

The STRING database was next used to construct a gene PPI network (Figure 5). After the isolated nodes were removed by Cytoscape 3.7.1, a PPI network of shared DEGs between GSE80178, GSE68183 and GSE43950 was generated, which contained 6 hub genes (Figure 5).

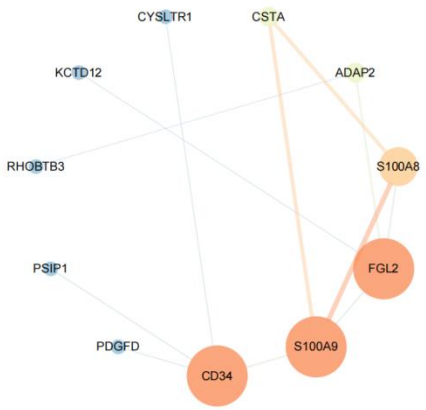


Figure 5: PPI network of shared DEGs between GSE80178, GSE68183 and GSE43950

4. Discussion

DFU is one of the most serious complications of DM, with high recurrence rate, amputation rate and mortality rate, which brings a serious burden to patients and society. DFU wounds usually show the characteristics of ischemia, hypoxia and chronic inflammation[3], which are difficult to cure[4,5]. Although researchers have carried out some studies on the pathogenesis of DFU, but the understanding of it is still relatively limited. Therefore, it is necessary to have a deeper understanding of its pathogenesis in order to find potential treatment targets and provide more treatment options.

In this study, the gene expression profile of GSE80178, GSE68183, GSE43950 were downloaded and analyzed by bioinformatics methods. Finally, 14 shared DEGs between DFU and diabetic microvascular disease were identified for

subsequent GO functional enrichment. Enrichment analysis showed that these genes were mainly associated with chronic inflammation, cell migration, immune response and extracellular matrix, which containing chronic inflammatory response, leukocyte migration, cellular transition metal ion homeostasis, vascular wound healing, collagen-containing extracellular matrix and Toll-like receptor binding. These annotation results provided valuable clues to reveal molecular interactions in the development of DFU. Clinical and experimental evidence suggests that DFUs fail to follow an orderly and selflimited progression of healing events and are characterized by a sustained inflammatory phase, which leads to permanent residence of inflammatory cells in the wound microenvironment, thereby chronically upregulating proinflammatory cytokines and transforming wounds into nonhealing chronic wounds[6]. Liu et al. reported that enhanced neutrophil extracellular traps in diabetic wounds triggered nod-like receptor protein 3 (NLRP3) inflammasome activation and IL-1β release in macrophages via the toll-like-receptor TLR-4/TLR-9 signaling pathway which are involved in the sustained inflammatory phase in DFUs[7]. Chang et al. found that monocytes come to the DFU and become macrophages (a type of white blood cells that digest microbes and cell debris through phagocytosis) and secrete transforming growth factor (TGF)-β1 and vascular endothelial growth factor (VEGF) for angiogenesis, results in production of collagen, hyaluronic acid, and fibronectin to form the new extracellular matrix[8]. As shown in the KEGG pathway analysis， genes were enriched in pathways such as “IL-17 signaling”, “glycosaminoglycan degradation” and “calcium signaling”. It is suggested genes or pathways relating to pro-inflammatory cytokines, glycosaminoglycan degradation and calcium channel might have important roles in the pathophysiology of DFU.

In order to further explore the potential hub genes between DFU and diabetic microvascular disease, we also screened the hub gene through PPI analysis. The results show that S100A9, S100A8, CSTA, ADAP2 (upregulation) and CD34, FGL2 (downregulation) may be the hub gene whose plays a pivotal role in DFU. In physiological function, S100A8 and S100A9 can be combined into homodimers or to bind in a non-covalent to form a willing dimer. When the concentration of Ca^{2+} changes, they also have other trimer or tetramer forms, and participate in the regulation and metabolism of cytoskeleton, arachidonic acid metabolism and anti-infective immune process in different ways. S100A8 and S100A9 can also recruit inflammatory cytokines to participate in anti-inflammatory response[9,10], so it has a certain value to reflect the intensity of inflammatory response. Singh et al. reported that the upregulation of bone marrow-derived pro-inflammatory cytokine S100A8 is one of the mechanisms leading to wound healing disorders and chronic ulcers in T2DM subjects[11]. ADAP2 is a member of the protein family with ADP ribose factor GTP enzyme activating protein domain. The protein encoded by this gene can bind to beta- tubulin and increase the stability of microtubules. It has been proved to be an important interferon stimulating gene in the immune system[12].CD34 is a transmembrane protein that is selectively expressed on the surface of mammalian hematopoietic stem or progenitor cells. When the cells gradually mature, the expression of CD34 decreases until it disappears. It is one of the main indicators for monitoring the microvessel density of new blood vessels[13], and is closely related to the biological process vascular wound healing. The downregulation of this gene may be related to the long-term nonunion of DFU. CSTA is a cysteine protease inhibitor that inhibits a variety of proteases, such as papain and cathepsin B, H and L, and is involved in cell adhesion, epidermal development and maintenance[14]. FGL2 is a membrane protein with prothrombinase at the N-terminal, expressed in a variety of cells, such as macrophages, dendritic cells, and endothelial cells, and can be induced robustly and exclusively in macrophages in response to stimulation with cytokines (IFN or TNF-α), viral infection, and lipopolysaccharide[15]. This suggests that FGL2 itself is a critical mediator of inflammation in that the interaction between inflammation and coagulation is reciprocally exacerbated in terms of inflammatory cytokine production and tissue factor secretion[16]. However, the role of CSTA and FGL2 in DFU needs to be further studied.

5. Conclusion

In this study, we analyzed the data sets of DFU and diabetic microvascular disease by bioinformatics, the co-expression

genes were identified and their functions were analyzed. It is shown that S100A9, S100A8, ADAP2 and CD34 are potential biomarkers for predicting DFU, which can provide ideas for follow-up research and treatment.This study still has some limitations, such as the small number of samples and the lack of clinical sample verification. More in-depth experiments are needed to verify the results of this study in the future.

References

[1] Sun H, Saeedi P, Karuranga S, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Research and Clinical Practice, 2022, 183: 109119.

[2] Cruciani M, Lipsky BA, Mengoli C, et al. Granulocyte-colony stimulating factors as adjunctive therapy for diabetic foot infections. Cochrane Database of Systematic Reviews, 2013, 8(8).

[3] Vieceli Dalla Sega F, Cimaglia P, Manfrini M, et al. Circulating Biomarkers of Endothelial Dysfunction and Inflammation in Predicting Clinical Outcomes in Diabetic Patients with Critical Limb Ischemia. Int J Mol Sci. 2022, 23(18):10641.

[4] Catrina SB, Zheng X. Hypoxia and hypoxia-inducible factors in diabetes and its complications. Diabetologia. 2021, 64(4):709-716.

[5] Deng L, Du C, Song P, et al. The Role of Oxidative Stress and Antioxidants in Diabetic Wound Healing. Oxid Med Cell Longev. 2021, 2021:8852759.

[6] Yang S, Hu L, Han R, et al. Neuropeptides, Inflammation, Biofilms, and diabetic Foot Ulcers. Exp Clin Endocrinol Diabetes. 2022, 130(7):439-446.

[7] Liu D, Yang P, Gao M, et al. NLRP3 activation induced by neutrophil extracellular traps sustains inflammatory response in the diabetic wound. Clin Sci (Lond). 2019, 133(4):565-582.

[8] Chang M, Nguyen TT. Strategy for Treatment of Infected Diabetic Foot Ulcers. Acc Chem Res. 2021, 54(5):1080-1093.

[9] Argyris PP, Slama Z, Malz C, et al. Intracellular calprotectin (S100A8/A9) controls epithelial differentiation and caspase-mediated cleavage of EGFR in head and neck squamous cell carcinoma. Oral Oncol. 2019, 95:1-10.

[10] Pruenster M, Vogl T, Roth J, et al. S100A8/A9: From basic science to clinical application. Pharmacol Ther. 2016, 167:120-131.

[11] Singh K, Agrawal NK, Gupta SK, et al. Increased expression of TLR9 associated with pro-inflammatory S100A8 and IL-8 in diabetic wounds could lead to unresolved inflammation in type 2 diabetes mellitus (T2DM) cases with impaired wound healing. J Diabetes Complications. 2016, 30(1):99-108.

[12] Bist P, Kim SS, Pulloor NK, et al. ArfGAP Domain-Containing Protein 2 (ADAP2) Integrates Upstream and Downstream Modules of RIG-I Signaling and Facilitates Type I Interferon Production. Mol Cell Biol. 2017, 37(6):e00537-16.

[13] Marcianò A, Ieni A, Mauceri R, et al. CD34 and CD105 Microvessels in Resected Bone Specimen May Implicate Wound Healing in MRONJ. Int J Environ Res Public Health. 2021, 18(21):11362.

[14] Blaydon DC, Nitoiu D, Eckl KM, et al. Mutations in CSTA, encoding Cystatin A, underlie exfoliative ichthyosis and reveal a role for this protease inhibitor in cell-cell adhesion. Am J Hum Genet. 2011, 89(4):564-71.

[15] Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. Cell Mol Immunol. 2016, 13(3):316-27.

[16] Ju C, Reilly TP, Bourdi M, et al. Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. Chem Res Toxicol. 2002, 15(12):1504-13.