Research Article Molecular signatures in diabetic foot ulcer by integrated gene expression profiling via bioinformatic analysis

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(Received: May 2022 Revised: June 2022 Accepted: July 2022)

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ABSTRACT

Introduction and Aim: Diabetic foot ulcers (DFUs) are a common and debilitating diabetic consequence leading to lower-limb amputations, long-term disability, and reduced lifespan. Hence, the current research aims to find out how differently expressed genes (DEGs) affect the DFU.

Materials and Methods: Bioinformatics analysis was used to evaluate DEGs using the GSE132187 dataset of the NCBI-GEO database, which contained samples from three hyperglycemic and three normoglycemic macrophage-like cell lines. Gene Ontology (GO) and KEGG pathway enrichment analysis was used to study how genes are classified into preset bins based on their functional properties after DEGs were discovered. A network of protein-protein interaction (PPI) was created and five topological characteristics such as degree, stress, closeness centrality, betweenness centrality, and radiality were evaluated to uncover hub DEGs in DFU.

Results: We found 547 DEGs using the GSE132187 dataset, comprising 79 upregulated DEGs and 468 downregulated DEGs. In total, the PPI network included 434 nodes and 1724 edges. The giant network uncovered six modules that are significantly enriched in biological processes like regulation of positive JNK cascade, positive interferon-gamma production and negative cell proliferation, cellular response to zinc ion and lipopolysaccharide, wound healing, and inflammatory response.

Conclusion: Bioinformatics analysis revealed the major differentially expressed hub-genes implicated in DFUs. These findings suggest that these genes could be exploited as DFU prognostic, diagnostic, or therapeutic targets.

Keywords: Bioinformatics analysis; diabetic foot ulcer; differentially expressed genes; inflammatory molecules.

INTRODUCTION

iabetes mellitus (DM) is a long-term endocrine disease characterized by hyperglycemia and abnormally high glucose levels. The core causes of DM are a lack of insulin secretion, changed insulin levels, and beta-cell death, which leads to a metabolic imbalance (1). Adults with diabetes account for around 537 million worldwide and is more common in the middle-aged and older population (2). The most life-threatening diabetes complication is the diabetic foot ulcer (DFU), which affects 15-25 % of diabetics and by raising morbidity and hospitalization rates, DFUs inflict a huge burden on society and families (3). Diabetics are more prone to develop macro and microvascular complications, decreased angiogenesis, infection, extracellular matrix changes, nephropathy and neuropathy (4, 5). The bulk of them are typically detected in DFUs, although it's unclear whether they have a causal or consequential role in healing impairment. A good comprehension of the etiologies and molecular basis of DFU is designed to establish more therapeutically effective medicines (6). However, these studies simply focus on one chemical, gene, or pathway but, some proteins, for example may

interact with other proteins and hence play an important part in the DFU (7).

Recently, genome-wide technologies followed by network biology and bioinformatics approaches have been employed for DFU to find the key genes that could be targeted as predictive or diagnostic indicators, as well as therapeutics. The analysis of primary gene products has also been investigated as a diagnostic and screening tool for disease detection, using network-based approaches to unravel the molecular structure and function of complex biological pathways. Such approaches try to investigate all gene products at the same time in order to gain a better knowledge of disease causes and discover effective intervention targets. As a result, in the current research we downloaded an original microarray dataset GSE132187 and analyzed it to find DEGs between macrophage-like cell lines in hyperglycemic and normoglycemic conditions. To discover the specific genes and its existing biological mechanisms, development processes, and to evaluate the network of protein-protein interactions (PPI), we employed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

analysis. Overall, integrated bioinformatics techniques were used to detect appropriate inflammatory signals in DFUs, which would identify potential biomarkers for diagnosis and also help to develop targeted therapeutics.

MATERIALS AND METHODS

Microarray dataset

The Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo) gathers and disseminates high-throughput genome-based data obtained by microarrays, next-generation sequencing (NGS), and other methods (8). We extracted the gene expression dataset #GSE132187, which was run on the Affymetrix U133 Plus 2.0 Array platform #GPL26734. The probes in the platform were transformed into suitable gene codes based on the annotation knowledge. Three macrophage-like cell lines housed in hyperglycemic settings and three macrophage-like cell lines kept in normoglycemic conditions were included from the GSE132187 data set.

Differentially Expressed Genes (DEGs) identification

The GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) tool was used to detect the differentially expressed genes (DEGs) using a |logFC| of at least 1 and a p<0.05. The DAVID program was used to transform the differential gene original codes filtered and processed by GEO2R into an official gene symbol (9).

Gene ontology and KEGG pathway enrichment analysis

A large set of genes collected from previous genetic studies were investigated using the tool DAVID, (Database for annotation, visualization, and integrated

Discovery(https://david.ncifcrf.gov/summary.jsp) (10). The GO (http://www.geneontology.org/) creates vocabularies and categories that can be used to annotate genes, gene products, and sequences (11). Based on the molecular, chemical and systemic functions, the KEGG (http://www.genome.jp/kegg/) develops biological science (12). All DEGs were analyzed for functional and pathway enrichment by DAVID software. For the screening of prominent GO keywords and KEGG pathways, a statistically significant difference was defined as< 0.05.

Construction of PPI network and module analysis

Online databases that search for interacting genes, such as STRING (http://string-db.org) (v11.0), are used to predict PPI networks (13). The study of protein functional interactions may uncover the mechanisms that underlying disease initiation and progression. In the current research, the PPI structure of candidate genes was built with a confidence level score of >0.4. Cytoscape is a bioinformatics software platform for testing interactive cellular networks (v3.3.0) (14). MCODE (Molecular Complex Detection) (v1.3.2) is a Cytoscape plug-in that cluster networks as per morphology in attempt to discover fully connected parts (15). The PPI network is depicted in Cytoscape, whereas MCODE indicates the most essential module. The selection criteria were MCODE scores >5, node score cut-off = 0.2, max depth = 100, and k-score =4. The Cytoscape plug-in Network Analyzer is used to do a topological analysis of the interaction network.

Selection of Hub-genes

CytoHubba, one of the Cytoscape plugin was used to select the top hub-genes in the PPI network. We used five methods to select the five most common genes: degree, stress, closeness centrality, betweenness centrality, and radiality (16).

RESULTS

Identification of DEGs

Using the aforementioned cutoff (|logFC| 2 and p<0:05), 547 DEGs (79 up regulated and 468 down regulated) were screened from the GSE132187 dataset with GEO2R analysis (Fig.1a-c). The network's shortest paths will connect two randomly selected nodes, and the distribution of shortest path length, betweenness centrality, closeness centrality, degree, average clustering coefficient, topological coefficient, and average neighborhood connectivity of network nodes was plotted using histograms (Fig.2a-h). The top-five overlapping genes were picked based on the five classification strategies in cytoHubba and found to belong to the down regulated DEGs network (Fig.3 and Table 1).

GO and KEGG pathway enrichment analysis of downregulated DEGs

According to the GO Enrichment analysis, the down regulated DEGs were substantially more abundant in biological processes (BP) such as keratinocyte migration, replicative cell ageing, positive control of fever creation, isocitrate metabolic process, production, and interleukin-1 beta neutrophil homeostasis. For the cell component (CC), DEGs were discovered in the mitochondrial respiratory chain complex I, peroxisome, mitochondrial large ribosomal subunit, intermediate filament cytoskeleton, and the lysosomal membrane's luminal side. The down regulated DEGs were involved in the molecular functions (MF) such as NADPH binding receptor agonist activity, CXCR chemokine receptor and cofactor binding, oxidoreductase activity, action on CH-OH group of donors, NAD or NADP as acceptor 1-phosphatidylinositol binding, isocitrate dehydrogenase (NADP+) activity, and pyruvate dehydrogenase activity. According to the KEGG pathway enrichment analysis, the DEGs were richly associated with glutathione metabolism, complement and coagulation cascades, hematopoietic cell lineage and inflammatory bowel disease.

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Fig. 1: Core network or giant network (a), up regulated (b), and down regulated (c) DEGs obtained from GEO2R analysis

| Table 1: | Top | five hub- | genes scores f | for degree, | betweenness | centrality, | , and closenes | s centrality |
|----------|-----|-----------|----------------|-------------|-------------|-------------|----------------|--------------|
|----------|-----|-----------|----------------|-------------|-------------|-------------|----------------|--------------|

| Gene ID | Gene name | Degree | BC | СС |
|----------------------------------|--------------------|--------|----------|----------|
| IL6 | Interleukin-6 | 77 | 0.111629 | 0.411206 |
| TNF Tumor necrosis factor | | 73 | 0.124314 | 0.411206 |
| HRAS | H Ras Protein | 57 | 0.146357 | 0.411597 |
| FGF2 Fibroblast growth factor- | | 41 | 0.04635 | 0.371674 |
| IL1B | Interleukin 1 beta | 39 | 0.014306 | 0.36204 |



Fig. 2: Essential genes in diabetic foot ulcers: a topological analysis of PPI network. a) The histogram depicts the shortest path length distribution, which shows the small-world property of the network (a), number of shared neighbors (b), betweenness centrality (c), closeness centrality (d), average clustering coefficient of network nodes (e), average neighborhood connectivity of network nodes (f), degree distribution of network nodes (g) and topological coefficient of network nodes (h).

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Fig.3: Five hub-genes were identified by overlapping the five classification methods like degree, stress, betweenness centrality, closeness centrality and radiality of cytoHubba.



Fig. 4: Cytoscape showing the gene modules and unclustered genes. Genes belonging to each module are highlighted in distinct colours for each distribution. The grey-coloured genes are unclustered

PPI network development and module analysis

After hiding nodes that couldn't communicate with other nodes, the PPI network had 434 nodes and 1724 edges. We then created a total of six modules using MCODE to perform K kernel analysis on the string network (Table 2 and Fig.4). The DEGs from this huge network were clustered into six modules M1-M6. In the BP, chemokine-mediated signalling pathway, inflammatory and immune mechanisms and were all highly enriched in DEGs for the Module 1 (M1). Modules for the CC, DEGs were identified at high amounts in the plasma membrane, extracellular space, and mitochondrial inner membrane. DEGs

were predominant in chemokine receptor binding, Binding of the CCR1 chemokine receptor, cytokine activity, and CXCR chemokine receptor binding in terms of MF. It was mostly related with cytokinecytokine receptor interaction in the KEGG pathway study biological processes like such as positive modulation of interleukin-6 production, positive management of interferon-gamma production, inflammatory response, and cellular response to tumour necrosis factor are enriched in Module 4 (M4). DEGs for the cell component were most likely located in the transcription factor complex, nucleolus, and extracellular space. DEGs were involved in cytokine activity, action of growth factors and interleukin-6 receptor binding when it came to MF. In the KEGG pathway research, it was strongly connected to the TNF signalling pathway. Biological processes such as mitochondrial translational termination, mitochondrial translational elongation, translation, and positive control of endothelial cell proliferation were considerably enriched in Module 6 (M6). Significant results were not seen by CC and MF. However, in module 6 there is no specific KEGG pathway. Similarly, in module 2, 3 and 5 GO function enrichment and specific KEGG pathways are not seen.

DISCUSSION

The low levels of TNFa cytokine is known to promote inflammation but it inhibits the formation of extracellular matrix at high levels (17). In addition, it can stimulate the acute-phase response, operate as a powerful neutrophil chemo-attractant and stimulate the macrophage activation when combined with $IL1\beta$ and IL6. TNFa is always increased in diabetics with acute hyperglycemia, adding to the general chronic inflammatory status seen in this condition. In hyperglycemia condition, IL6 concentration was found to be increased in macrophages of normal mice, streptozotocin-injected and db/db mice (18). The lack of IL6 receptor's α -subunit leads to impaired infiltration of macrophages and also delay in wound healing was observed in a mice study (19). The circulatory levels of acute-inflammatory responsive proteins and IL6 were considerably higher in diabetics with foot ulcers, compared to who did not have foot ulcers (20). Circulating monocytes and tissue macrophages produce IL1 β , an essential inflammatory molecule for the activated caspase-1 cleavage in the lysosome. By forcing the NALP3 inflammasome to assemble, IL-1 β can increase its own secretion. IL-1 levels are higher in DFU patients, but they decrease when the ulcers heal (21). Topical IL-1 therapy was linked to higher CXCR2 expression which results in delayed wound healing in skin explants. The macrophages isolated from wounds of diabetics and db/db mice show elevated IL1 β and components of NALP3 inflammasome. and blocking the inflammasome activation indicated the better healing in 10 days of recovery period (22).

Signals from outside the cell are sent to the cell's nucleus via signal transduction, which is carried out by the HRas protein. This signal promotes cell growth and division. The HRas protein, which also functions as a GTPase, converts a chemical called GTP to GDP. The HRAS gene, which was discovered in this study, had never been linked to DFUs before. As a result, confirming the functional importance and molecular roles of these genes in the DFU network is vital. In a few of studies, the hyperglycemia and the deposition of advanced glycation end products at the wound site have been related to tissue repair in diabetic skin (23). In addition, the local degradation of FGF2 and/or absorption into excipients, causing it to lose its effectiveness, hence incorporation it into medicated gels might have major influence on wound healing. The FGF2 integrated chitosan hydrogel application to wounds in db/db mice showed that there is lot of tissue granulation, capillary growth, and formation of epithelium (24).

| Modules | Nodes | Edge | MCODE Score | Gene IDs |
|---------|-------|------|-------------|---|
| M1 | 36 | 197 | 11.257 | UQCR10, PLAUR, NDUFB1, PLAU, ATP5L, NDUFB11, VAMP8, |
| | | | | CCL5, CD33, CXCL1, NDUFB7, CCL4, CXCL2, ATP5F1, CYBB, |
| | | | | CX3CR1, GPR18, COX6B1, CD36, IL1A, CCL19, CSF2, CXCL3, |
| | | | | UQCRQ, RAP2C, NDUFA6, NDUFB2, CD59, NDUFA11, GT, |
| | | | | LAIR1, CD300A, GAA, IL1B, ITGAL, HVCN1 |
| M2 | 7 | 21 | 7.000 | FBXO41, HERC4, ASB13, UBE2O, RNF7, UBE2D4, ANAPC13 |
| M3 | 16 | 52 | 6.933 | IDH1, IDH2, LDHB, MDH1, MDH2, MRPL48, MRPL55, |
| | | | | MRPS23, PDHA1, PDHB, PFKP, RPL3, RPS14, RPS20, |
| | | | | SUCLG1TKT |
| M4 | 24 | 75 | 6.522 | GM2A, CREG1, MRPS9, NHLRC3, IMPDH1, LIF, PARP1, |
| | | | | C3AR1, XIAP, MAP3K5, PYCARD, AGA, HRAS, IL6, PEBP1, |
| | | | | TNF, GUSB, THBD, HBEGF, CTSA, ITPKB, MRPL40, CLEC7A, |
| | | | | SMAD7 |
| M5 | 5 | 10 | 5.000 | MT1G, MT1F, MT1X, MT1E, SLC30A1 |
| M6 | 16 | 30 | 4.000 | HSPA8, PACSIN2, SECISBP2, ANG, MRPL24, VEGFC, RPL14, |
| | | | | FGF2, NHP2, SYNJ2, TIMP3, MMP7, MRPL12, MRPL17, SNX9, |
| | | | | WNT5A |

Table 2: DEGs associated with each cluster and their MCODE scores

Additionally, topical use of a mixture of aloe vera gel and ryegrass hydroalcoholic extract shortened the inflammatory phase by boosting FGF-2 and also accelerated the healing of diabetic open wounds by elevating cell viability and collagen deposition (25). To summarize, employing genomics to anticipate possible targeted therapy targets in diseases is a successful clinical research strategy, despite some drawbacks. A public dataset from GEO was used to look into the possible diagnosis and treatment targets of DFU. Current estimates of possible prophylactic and treatment targets must be confirmed using experimental methodologies in cell and animal models prior to clinical trials.

CONCLUSION

Overall, our bioinformatics network analysis of hub genes indicates that TNF α , IL6, IL1 β , FGF2, and HRAS as important hub-genes involved in DFU healing. However, these forecasts have to be confirmed with the biological investigations, but the key intriguing genes discovered could be helpful in gaining a better knowledge of wound recovery.

ACKNOWLEDGMENT

The authors are thankful to the Central Research Laboratory, K S Hegde Medical Academy, and the Registrar, NITTE (Deemed to be University), Mangaluru, India for providing all the support and facilities to complete this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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