Identification of hub genes and gene modules associated with Behçet's disease by weighted gene co-expression network analysis of neutrophil transcriptome

J. Zou[1], Y.R. Zhu[2], J.-L. Guan[1]

[1]Division of Rheumatology and Immunology; [2]Department of Dermatology, Huadong Hospital, Fudan University, Shanghai, China.

Abstract

Objective

Behçet's disease (BD) is a chronic inflammatory condition with recurrent skin lesions, uveitis, and oral and genital ulcers. Neutrophils are important in the pathogenesis of BD, but their molecular mechanisms are unclear.

Methods

We performed weighted gene co-expression network analysis on the transcriptome of neutrophils from 10 BD patients and 10 healthy controls to identify hub genes and gene modules associated with BD.

Results

We found eight co-expression modules with different biological functions. The turquoise module was involved in response to hydrogen peroxide and reactive oxygen species, the blue module was involved in response to external stimulus and inflammatory response, and the brown module was involved in the type I interferon signalling pathway. We further identified hub genes and transcription factors in each module by using module membership and gene significance.

Conclusion

Our results reveal novel gene modules and hub genes that are associated with neutrophil activation and dysfunction in BD, which could serve as potential biomarkers and therapeutic targets for this disease.

Key words

Behçet's disease, bioinformatics analysis, weighted gene co-expression network analysis, hub gene, neutrophils

Jun Zou, MD, PhD
Yi-Rong Zhu, MD
Jian-Long Guan, MD, PhD

Please address correspondence to:
Jun Zou
Division of Rheumatology and Immunology,
Huadong Hospital,
Fudan University,
no. 221 Yan'an West Road,
Shanghai 200040, China.
E-mail: drzoujun@163.com

and to:
Yi-Rong Zhu
Department of Dermatology,
Huadong Hospital,
Fudan University,
no. 221 Yan'an West Road,
Shanghai 200040, China.
E-mail: 88021821@qq.com

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Introduction

Behçet's disease (BD) is a chronic and systemic vasculitis with an unknown aetiology (1). It is characterised by recurrent oral and genital ulcers, uveitis, skin lesions, and involvement of various organs, such as the joints, nervous system, and vascular system. BD affects mainly young adults and has a higher prevalence in countries along the ancient Silk Road, such as Turkey, Iran, China, and Japan. The exact causes of BD are not completely comprehended. It is thought to incorporate both environmental elements along, with the disruption of the immune system (2).

Neutrophils, being the predominant category of leukocytes in humans, assume a pivotal function in the domain of innate immunity and inflammation (3, 4). Furthermore, they partake significantly in the pathophysiology of BD, as is evident from a multitude of studies that have disclosed escalated neutrophil activation, migration, and oxidative outbursts in BD patients (5). Moreover, neutrophils can release extracellular traps (NETs), which are web-like structures composed of DNA, histones, and granular proteins that trap and kill microbes. However, NETs can also cause tissue damage and autoimmunity by exposing self-antigens and inducing inflammatory cytokines. Previous studies have reported elevated levels of NETs and NET-related markers in BD patients, suggesting that NETs may contribute to the disease manifestations (6). To investigate the molecular mechanisms underlying neutrophil dysfunction in BD, we performed weighted gene co-expression network analysis (WGCNA) on peripheral blood neutrophils isolated from 10 BD patients and 10 healthy controls (HCs). WGCNA is a systems biology method that identifies clusters (modules) of highly correlated genes and relates them to phenotypic traits or clinical features (7). WGCNA has been widely used to study gene expression patterns in various diseases, such as cancer, neurodegenerative disorders, and autoimmune diseases. By applying WGCNA to BD neutrophils, we aimed to find the hub genes and gene modules that are associated with BD and its clinical characteristics. We also performed functional enrichment analysis and protein-protein interaction (PPI) network analysis to explore the biological functions and interactions of the hub genes and gene modules (8, 9). Our study provides new insights into the molecular basis of neutrophil aberrance in BD and may help to identify potential biomarkers or therapeutic targets for this disease.

Materials and methods

Data source and preprocessing

Utilising a publicly accessible dataset of Behçet's disease derived from treatment-naive BD patients, and age and sex-matched healthy volunteers. Major organ involvement includes: gastrointestinal involvement (1), ocular involvement (2), vascular involvement (6) and neurological involvement (1). The investigation was conducted on the blood transcriptome dataset [which is available in the GEO repository: GSE205867 dataset produced through the utilisation of the Illumina NovaSeq 6000 (a high-throughput sequencing platform) with the platform ID GPL24676] (5). This particular dataset encompassed the expression data of 10 individuals classified as healthy controls and 10 individuals diagnosed with Behçet's disease and unreated. For subsequent analysis, the data was examined through the utilisation of the "R" programming software (version 4.1.1). The Ethics Committee of the Huadong Hospital, Fudan University, approved this study

Weighted gene co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) is a systems biology method for describing the correlation patterns among genes across microarray samples (10). WGCNA can be used for finding clusters (modules) of highly correlated genes, for summarising such clusters using the module eigengene or an intramodular hub gene, for relating modules to one another and to external sample traits (using eigengene network methodology), and for calculating module membership measures.

We performed WGCNA on the normalised counts of all expressed genes us-

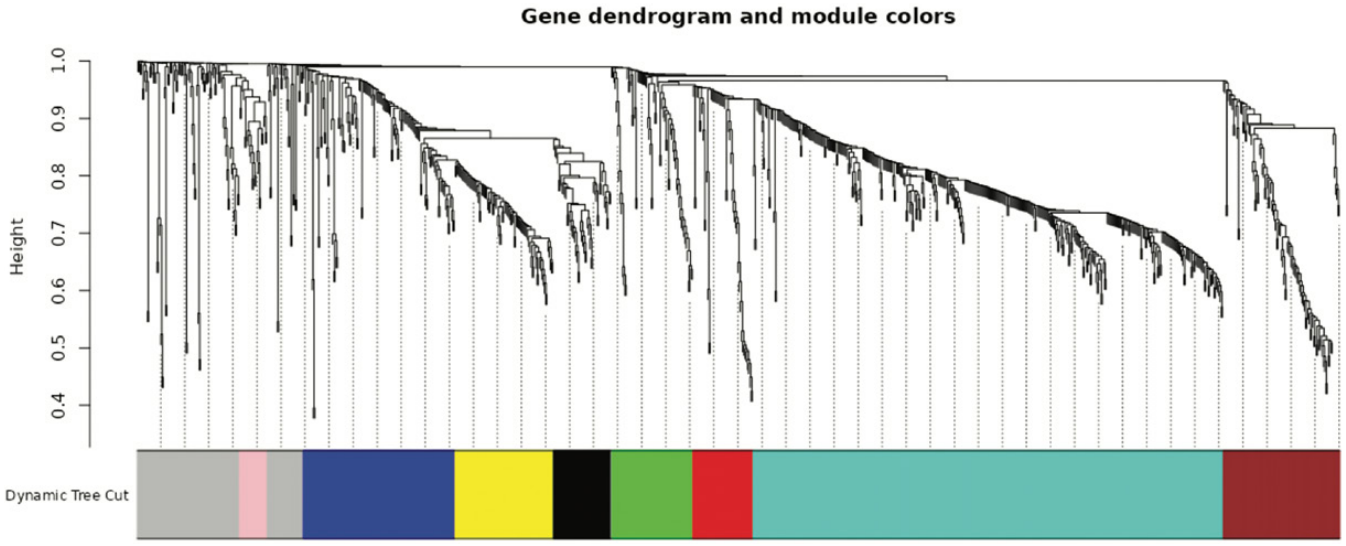

Fig. 1. The utilisation of a hierarchical clustering tree module aims to identify clusters (represented on the horizontal axes) of genes by considering the distance between them (represented on the vertical axes). The dynamic tree cut methodology reveals the division of the module based on the clustering results, while the merged dynamic approach signifies the division of the module based on the similarity observed within the module.

ing the WGCNA R package (10, 11). We first filtered out genes with low expression levels by setting a threshold of mean counts per million (CPM) >1 in at least three samples. Then, we calculated a matrix of Pearson's correlation coefficients for all pairs of genes across all samples, and transformed it into an adjacency matrix using a power function with a soft-thresholding parameter β=12, which was chosen to ensure a scale-free topology of the network. Next, we converted the adjacency matrix into a topological overlap matrix (TOM), which measures the network interconnectedness of two genes. Based on the TOM, we applied hierarchical clustering to group genes into modules using a dynamic tree-cutting algorithm with a minimum module size of 30 genes . We assigned each module a unique colour for identification and merged modules with high similarity (correlation >0.8) into larger modules.

In order to establish a connection between the gene modules and BD status, we conducted the calculation of two measurements: module significance (MS) and module membership (MM) (12). MS is characterised as the mean gene significance, denoting the absolute value of the correlation between gene expression and BD status, across all genes in a module. This measurement provides an overall indication of the association between a module and BD. On the other hand, MM is defined as the correlation between the module eigengene, which refers to the primary principal component of a module, and the gene expression profile. MM signifies the degree to which a gene aligns with a module. By identifying the modules with high MS (MS >0.5) and the genes with high MM (MM >0.8), we can pinpoint the most pertinent factors related to BD.

Functional enrichment analysis and protein-protein interaction network analysis

To explore the biological functions and pathways of the modules and genes associated with BD, we performed functional enrichment analysis using the clusterProfiler R package. We tested for overrepresentation of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in each module or gene set using hypergeometric tests (13, 14). We adjusted the *p*-values using the Benjamini and Hochberg's method and selected the terms or pathways with adjusted *p*-value <0.05 as significantly enriched.

To investigate the interactions among the genes associated with BD, we constructed protein-protein interaction (PPI) networks using the STRING database, which provides information on the physical and functional associations of proteins based on experimental evidence and computational prediction (15). We set a confidence score >0.4 as the cut-off criterion and visualised the PPI networks using Cytoscape software. We also calculated two topological parameters for each node (gene) in the network: degree centrality and betweenness centrality. Degree centrality is defined as the number of edges connected to a node and reflects the local importance of a node in the network. Betweenness centrality is defined as the fraction of shortest paths between any two nodes in the network that pass through a given node and reflects the global influence of a node in the network. We identified the nodes with high degree centrality (> mean + 2 standard deviations) or high betweenness centrality (> mean + 2 standard deviations) as hub nodes or bottleneck nodes, respectively.

Results

Construction and analysis of co-expression network

Weighted gene co-expression network analysis were performed to identify gene modules correlating with BD status. We constructed a co-expression network using 18,209 genes that passed the filtering criterion, 58,735 genes in 20 samples. Of the 35170 genes passed filter, 34,991 were converted to Ensembl gene IDs in our database. We detected eight modules of co-expressed

genes, which were assigned different colours for identification. The grey module contained genes that were not assigned to any other modules. The number of genes in each module ranged from 23 to 391. The module sizes and colours are shown in Figure 2A.

We performed calculations to determine the significance of each module, which denoted the average significance of genes within each module. Significance of genes was defined as the absolute value of the correlation between the expression of genes and the status of BD.

We also calculated the module membership for each gene in each module, which represented the correlation between the gene expression profile and the module eigengene. Module eigengene was defined as the first principal component of the standardised expression data of a given module, and represented the overall expression pattern of the module.

Weighted gene co-expression network analysis of BD neutrophils Dendrogram showes the clustering of genes into co-expression modules Each branch represents a gene, and each colour represents a module. The grey colour represents genes that are not assigned to any modules. Bar plot showing the module significance for each module Module significance is defined as the average gene significance across all genes in a module. Gene significance is defined as the absolute value of the correlation between gene expression and BD status.

Functional enrichment analysis of co-expression modules

To gain insights into the biological functions and pathways of the co-expression modules, we performed functional enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID). We selected the turquoise, brown, and blue modules as the most relevant to BD and used the whole gene list as the background. We obtained the enriched GO terms and KEGG pathways for each module with a false discovery rate (FDR) <0.05. The top 10 enriched terms or pathways for each module are shown in Table I. The turquoise module was mainly enriched in GO terms

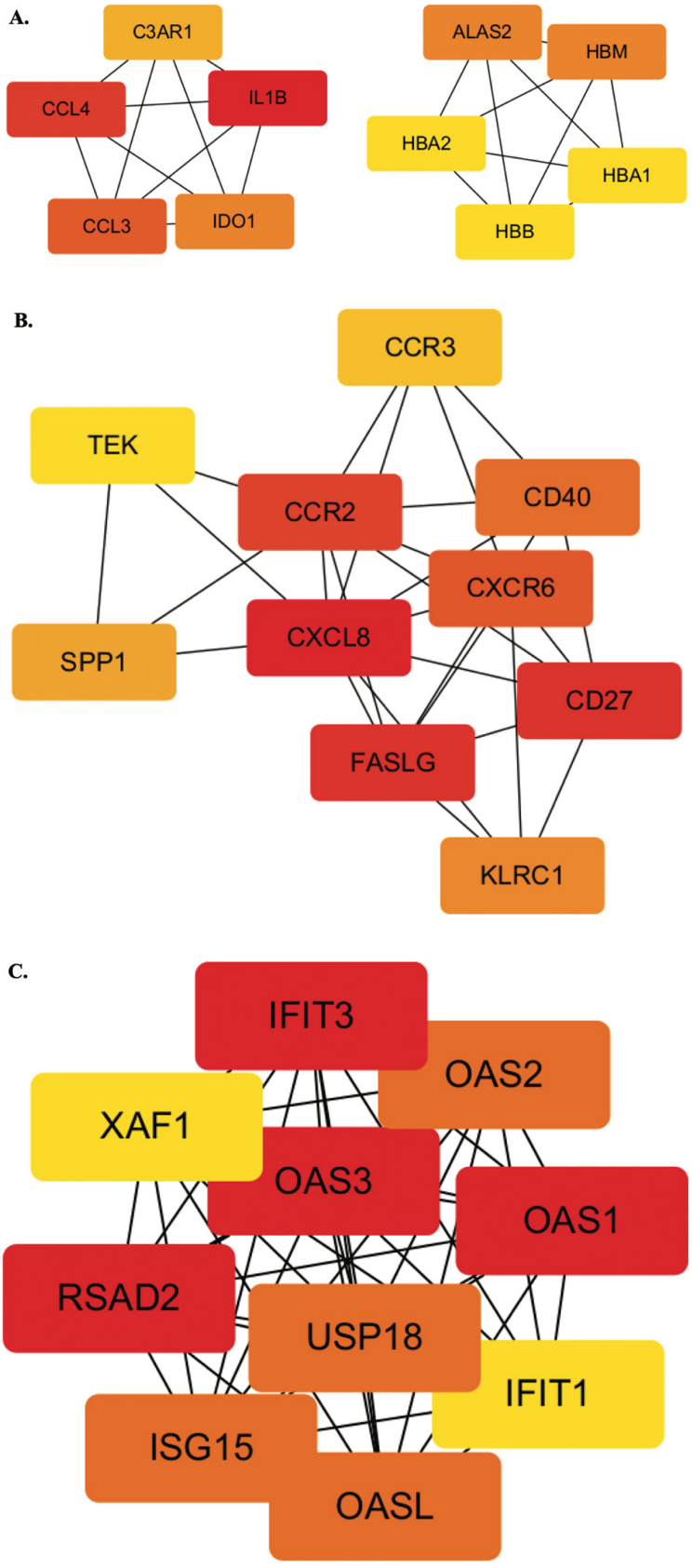

Fig. 2. A-C. The top 10 hub genes in three modules, were identified by the CytoHubba Cytoscape plugin. These hub genes are characterised by their extensive network connections. To visually depict their significance, the image employs a colour scale ranging from red, denoting the highest importance, to yellow, indicating a lower level of importance.

Table I. Functional enrichment analysis of turquoise modules (429 genes).

adj.Pval	Genes	Pathways
2.70E-06	5	Oxygen transport
7.30E-06	5	Gas transport
2.80E-05	5	Hydrogen peroxide catabolic process
1.80E-04	7	Response to hydrogen peroxide
1.60E-03	7	Response to reactive oxygen species
1.60E-03	4	Bicarbonate transport
1.60E-03	5	Lymphocyte chemotaxis
2.00E-03	5	Monocyte chemotaxis
3.10E-03	5	Chemokine-mediated signalling pathway
3.40E-03	5	Neutrophil chemotaxis
3.40E-03	9	Positive regulation of MAPK cascade
3.40E-03	2	Positive regulation of MAPKKK cascade by fibroblast growth factor receptor signalling pathway
3.40E-03	5	Cellular oxidant detoxification
3.40E-03	5	Response to chemokine
3.40E-03	5	Cellular response to chemokine

related to metabolic processes such as glycolysis, oxidative phosphorylation, and fatty acid degradation. The most enriched KEGG pathway was metabolic pathways, which indicated the high energy demand and metabolic activity of neutrophils. The brown module was mainly enriched in GO terms related to immune response, such as leukocyte activation, inflammatory response, and cytokine production. The most enriched KEGG pathway was cytokine-cytokine receptor interaction, which involved many pro-inflammatory cytokines and chemokines that regulate neutrophil function. The blue module was mainly enriched in GO terms related to the cell cycle, such as DNA replication, mitotic nuclear division, and chromosome segregation. The most enriched KEGG pathway was cell cycle, which reflected the proliferative capacity of neutrophils, Table I.

Identification of hub genes in expression modules

To identify the hub genes and transcription factors that were potentially involved in the regulation of BD neutrophils, we performed network analysis using the Cytoscape software. We imported the gene expression data and the co-expression modules into Cytoscape and constructed a network for each module. We used the cytoHubba plugin to rank the nodes in the network based on their degree centrality, which represented the number of connections a node had with other nodes. We selected the top 10 nodes with the highest degree of centrality as the hub genes for each module.

The set of hub genes in the turquoise module encompassed IL1B, CCL4, CCL3, HBM, IDO1, ALAS2, C3AR1, HBB, HBA2, and HBA1, whereas the hub genes in the blue module comprised CXCL8, CD27, FASLG, CCR2, CXCR6, CD40, KLRC1, SPP1, TEK, and CCR3. Lastly, the hub genes in the brown module consisted of OAS3, OAS1, RSAD2, IFIT3, USP18, OAS2, ISG15, IFIT1, and XAF1 (Table II-IV).

Identification of transcription factors involvement

To identify the transcription factors that regulate gene expression during human embryonic stem cell differentiation, we performed a transcription factor binding site (TFBS) motif enrichment analysis on the promoters of down-regulated genes. By comparing the frequency of TFBS motifs in the promoters of down-regulated genes to a background set of promoters, we identified the transcription factors that are involved in hESC differentiation and gene regulation. The most significant motifs were CCG and CG, which are bound by DNMT1 and MLL, respectively. Both of these transcription factors belong to the CxxC family, which recognises unmethylated CpG dinucleotides and regulates DNA methylation. DNA methylation is an epigenetic modification that affects gene expression and cell fate. The second most significant family was E2F, which consists of transcription factors that regulate cell cycle progression and apoptosis. Four E2F members were enriched in the down-regulated genes, suggesting that they may inhibit cell proliferation and promote differentiation. Another prominent family was C2H2 zinc finger (ZF), which comprises transcription factors that bind to various DNA sequences and regulate diverse biological processes. Seven C2H2 ZF transcription factors were enriched in the down-regulated genes, indicating that

Table II. Top 10 in network hub genes in the turquoise module ranked by maximum clique centrality (MCC) in CytoHubba.

Rank	Name	Score
1	*IL1B*	50
2	*CCL4*	36
3	*CCL3*	30
4	*HBM*	26
4	*IDO1*	26
4	*ALAS2*	26
7	*C3AR1*	25
8	*HBB*	24
8	*HBA2*	24
8	HBA1	24

Table III. Top 10 in network hub genes in the blue module ranked by maximum clique centrality (MCC) in CytoHubba.

Rank	Name	Score
1	*CXCL8*	107
2	*CD27*	78
2	*FASLG*	78
4	*CCR2*	66
5	*CXCR6*	56
6	*CD40*	40
7	*KLRC1*	24
8	*SPP1*	18
9	*CCR3*	14
10	*TEK*	12

Table IV. Top 10 in network hub genes in the brown module ranked by maximum clique centrality (MCC) in CytoHubba.

Rank	Name	Score
1	*OAS3*	4.96E+24
1	*OAS1*	4.96E+24
1	*RSAD2*	4.96E+24
1	*IFIT3*	4.96E+24
5	*USP18*	4.96E+24
5	*OAS2*	4.96E+24
5	*ISG15*	4.96E+24
8	*IFIT1*	4.96E+24
9	*XAF1*	4.96E+24
10	HERC5	4.96E+24

they may modulate gene expression in response to external stimuli or developmental signals. Other transcription factor families that were enriched in the down-regulated genes included basic helix-loop-helix (bHLH), and activator protein-2 (AP-2).

Discussion

In this study, we utilised WGCNA to examine the neutrophil transcriptome data from BD patients and controls, aiming to discover the hub genes and gene modules related to BD. Although neutrophils are thought to play a role in the development of BD, their molecular mechanisms of activation and dysfunction remain unclear. WGCNA enabled us to create a co-expression network with seven modules and identify the hub genes and transcription factors in each module. Additionally, functional enrichment analysis was conducted to uncover the biological pathways and processes overrepresented in each module. Our study provides new insights into the molecular mechanisms of neutrophil activation and dysfunction in BD, suggesting potential biomarkers and therapeutic targets for this disease.

Hub genes were selected in each module based on their module membership and gene significance. Module membership is the correlation between the gene expression profile and the module eigengene, which represents the overall expression pattern of the module. Gene significance is the correlation between the gene expression and BD status, indicating the gene's relevance to the disease. Hub genes with high module membership and gene significance were chosen, implying their representation of the module and association with the disease.

The turquoise module hub genes consisted of IL1B, CCL4, CCL3, HBM, IDO1, ALAS2, C3AR1, HBB, HBA2, and HBA1. These genes were involved in hydrogen peroxide response, gas transport, and reactive oxygen species response. IL1B is a pro-inflammatory cytokine that regulates inflammation and immunity (16). CCL4 and CCL3 are chemokines that recruit immune cells to sites of inflammation (17). IDO1 is an enzyme that breaks down tryptophan, an important amino acid for T cell function (18). ALAS2 is an enzyme that starts haemoglobin biosynthesis and is crucial for hemoglobin synthesis and function (19). C3AR1 is a receptor for complement component C3a, involved in inflammation and immune responses (20). These hub genes suggest that neutrophils in BD patients have abnormal oxygen metabolism and hemoglobin function, potentially impairing their ability to handle oxidative stress and tissue hypoxia.

The blue module hub genes were CXCL8, CD27, FASLG, CCR2, CXCR6, CD40, KLRC1, SPP1, TEK, and CCR3. These genes were involved in responding to stimuli, causing inflammation, and sensing pain. CXCL8 attracts neutrophils to inflamed areas (21). CD27 modulates T cell activation and differentiation (22). FASLG induces apoptosis in target cells (23). CCR2 and CXCR6 guide the movement of monocytes, macrophages, and T cells (24). CD40 controls B cell activation and antibody production (25). KLRC1 recognises stress-induced molecules on target cells (26). SPP1 and CCR3 regulate cell adhesion, migration, and differentiation (27, 28). These hub genes indicate that BD patients' neutrophils have increased inflammatory and immune responses, potentially causing tissue damage and pain.

The brown module hub genes included OAS3, OAS1, RSAD2, IFIT3, USP18, OAS2, ISG15, IFIT1, and XAF1, which were involved in the type I interferon signalling pathway, cellular response to type I interferon, and defense response to virus DNA replication. These genes encode proteins that have antiviral and immunomodulatory effects by activating a complex signalling cascade. For example, OAS3, OAS1, OAS2, and ISG15 are interferon-stimulated genes that synthesise 2'-5' oligoadenylates, which activate RNase L to degrade viral RNA (29, 30). RSAD2 and IFIT1 are also interferon-stimulated genes that inhibit viral replication by targeting viral proteins or RNA (31). USP18 is a negative regulator of type I interferon signalling that protects cells from excessive inflammation (32). XAF1 is a pro-apoptotic protein that is induced by type I interferon and mediates cell death in response to viral infection (33). These hub genes suggest that neutrophils from BD patients have enhanced type I interferon signalling pathway, which may trigger a dysregulated immune system.

Our study used a publicly accessible dataset of neutrophils derived from treatment-naive BD patients and healthy volunteers (5). We have confirmed that none of the patients in this study were receivin g type I interferon therapy. The same dataset was previously analyzed by using a conventional gene expression analysis to dientify differentially expressed genes (DEGs), Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) and transcription regulatory network, revealed the predominant influence of the NF-κB pathway and chemotaxis. Differing from those results, we conducted WGCNA on neutrophil transcriptome data from BD patients and controls. We found eight co-expression modules associated with BD and characterised the hub genes, transcription factors, and biological functions of each module. Neutrophils from BD patients showed altered oxygen metabolism, hemoglobin function, enhanced inflammatory and immune responses, and activated type I interferon signalling pathway and antiviral responses. This discrepancy may be attributed to differences in data preprocessing, and statistical methods. Nonetheless, both studies highlight the involvement of neutrophils in BD pathogenesis and suggest a role for neutrophils in BD pathogenesis and potential biomarkers or therapeutic targets.

Several previous genetic studies have identified susceptibility genes for BD, some of which are associated with neutrophil dysfunction (34). The HLA-B51 allele, which is significantly correlated with BD, has been associated with modified neutrophil function, encompassing heightened generation of reactive oxygen species and pro-inflammatory cytokines (35). Another BD-associated gene, IL23R, is involved in the regulation of neutrophil migration and activation (36). Our study identified several hub genes that are also associated with neutrophil function and dysfunction.

Pro-inflammatory cytokines like IL1B, CCL4, and CCL3 can activate neutrophils and promote inflammation. CXCL8 is a potent neutrophil chemoattractant, and its increased expression in BD patients' neutrophils may contribute to the excessive neutrophil infiltration observed in this disease. These findings suggest that the hub genes identified in our study may play a role in the pathogenesis of BD, and further studies are needed to investigate their specific contributions.

The identification of hub genes and gene modules associated with BD provides potential targets for therapeutic intervention. Targeting the type I interferon signalling pathway, which is known to have both beneficial and detrimental effects in various diseases, could be a promising therapeutic strategy. Furthermore, the manipulation of the inflammatory and immune reactions controlled by key genes like IL1B, CCL4, and CXCL8 may also offer a viable method for treatment. Further studies are warranted to investigate the specific roles of these hub genes in BD pathogenesis and to develop targeted therapies.

Our investigation possesses a number of constraints. Initially, the magnitude of the sample is comparatively diminutive, which has the potential to impede the applicability of our discoveries. Additionally, the investigation is conducted in a cross-sectional manner, thereby prohibiting the establishment of a causal association between the identified central genes and BD. Lastly, the verification of the central genes' involvement in BD pathogenesis through functional validation experiments was not undertaken. In order to substantiate and broaden our discoveries, further investigations encompassing larger sample sizes, longitudinal designs, and functional experiments are imperative.

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