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ORIGINAL ARTICLE / ÖZGÜN MAKALE

UNVEILING THERAPEUTIC TARGETS THROUGH PATHWAY ANALYSIS AND IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN ULCERATIVE COLITIS

ÜLSERATİF KOLİTTE YOLAK ANALİZİ VE FARKLI İFADE EDİLEN GENLERİN BELİRLENMESİ YOLUYLA TERAPÖTİK HEDEFLERİN AÇIĞA ÇIKARILMASI

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ABSTRACT

Objective: *This study utilizes integrated bioinformatics to investigate Differentially Expressed Genes (DEGs) and pathways related to ulcerative colitis (UC).*
Material and Method: *Differentially Expressed Genes were identified from UC patients' colonic mucosal samples and controls using GSE13367 and GSE134025 datasets. Differentially Expressed Genes selection utilized GEO2R and Venn diagrams, followed by functional annotation, pathway analysis, PPI determination via the STRING database, and GO/KEGG enrichment analysis using Metascape.*
Result and Discussion: *Analysis unveiled 197 DEGs, with 76 up-regulated and 121 down-regulated genes. Up-regulated genes were enriched in humoral immune response, peptidoglycan binding, and NADPH oxidase complex, while down-regulated genes were linked to inorganic anion transport, transmitter-gated ion channel activity, and integral plasma membrane components. In the PPI network, up-regulated DEGs formed a dense network (75 nodes, 190 edges), indicating significant interactions, whereas down-regulated DEGs formed a less dense network (114 nodes, 63 edges). Five hub genes (CXCR4, CXCL13, CXCL1, MMP3) were identified among the 197 DEGs. These findings provide new insights into UC's causes and offer promise for more effective therapeutic approaches.*
Keywords: *Bioinformatic analysis, gene expression, inflammatory bowel disease, pathway enrichment analysis, protein interactions*

ÖZ

Amaç: *Bu çalışma, ülseratif kolit (ÜK) ile ilişkili DEG'leri ve yolları araştırmak için entegre biyoinformatik kullanır.*
Gereç ve Yöntem: *DEG'ler, GSE13367 ve GSE134025 veri kümelerini kullanarak ÜK hastalarının*

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kolonik mukozal örneklerinden ve kontrollerden belirlendi. DEG seçimi için GEO2R ve Venn diyagramları kullanıldı, ardından fonksiyonel anotasyon ve yol analizi yapıldı. Protein-protein etkileşimleri (PPI'ler) STRING veritabanı kullanılarak belirlendi ve Metascape, Gen Ontolojisi (GO) ve Kyoto Genler ve Genomlar Ansiklopedisi (KEGG) zenginleştirme analizi için kullanıldı.

Sonuç ve Tartışma: *Analiz, 197 DEG ortaya koydu, bunların 76'sı yukarı regüle edilmiş ve 121'i aşağı regüle edilmiş genlerdi. Yukarı regüle edilmiş genler, humoral immün yanıt, peptidoglikan bağlanma ve NADPH oksidaz kompleksi gibi süreçlerde zenginleşmişti. Aşağı regüle edilmiş genler, inorganik anyon taşıma, alıcı-gated iyon kanal aktivitesi ve integral plazma membran bileşenleri ile ilişkilendirildi. PPI ağındaki yukarı regüle edilmiş DEG'ler, 75 düğüm ve 190 kenarla yoğun bir ağ oluşturdu, önemli etkileşimleri gösterirken, aşağı regüle edilmiş DEG'ler, 114 düğüm ve 63 kenarla daha az yoğun bir ağ oluşturdu. 197 DEG arasında beş merkezi gen (CXCR4, CXCL13, CXCL1, MMP3) tanımlandı. Bu bulgular, ÜK'nin nedenleri hakkında yeni içgörüler sunmakta ve daha etkili tedavi yaklaşımları için umut vaat etmektedir.*

Anahtar Kelimeler: *Biyoinformatik analizi, gen ifadesi, inflamatuar bağırsak hastalığı, yol zenginleştirme analizi, protein etkileşimleri*

INTRODUCTION

Ulcerative colitis (UC) is a chronic subtype of inflammatory bowel disease characterized by extensive inflammation of the colonic mucosa. Several factors can influence the development of ulcerative colitis, including lifestyle choices such as stress, drug usage, diets high in sugar and fat, and smoking. Genetic factors also play a role in susceptibility to UC [1]. Additionally, immune regulatory disorders, as well as the continuous stimulation of antigens by commensal enteric bacteria, fungi, and viruses, can contribute to chronic inflammation in individuals with genetic abnormalities and impaired mucosal barrier function. The primary clinical symptoms of UC include abdominal discomfort and diarrhea with blood and mucus [2].

Identifying differentially expressed genes (DEGs) in the colonic mucosa that exhibit altered expression patterns in UC patients compared to healthy individuals can provide valuable insights into the disease. Advances in gene chip technology have generated substantial amounts of data on gene expression profiles [3]. To gain a deeper understanding of the pathophysiology of ulcerative colitis, this study used an integrated bioinformatics analysis to examine the DEGs associated with UC and their related pathways. This approach offers a comprehensive view of the molecular mechanisms involved in the disease, facilitating further research and potential therapeutic interventions [4].

MATERIAL AND METHOD

DEGs Identification from Datasets GSE13367 and GSE134025

DEGs (Differentially Expressed Genes) were identified in normal samples regarding Ulcerative Colitis (UC) using GEO2R, a collaborative digital tool available at http:// www.ncbi.nlm.nih.gov/geo/geo2r [5]. This software adopts a powerful algorithm to help identify DEGs of multiple experimental conditions by pairing datasets from the Gene Expression Omnibus (GEO) series. Data on gene expression profiles including GSE13367 and data from the GSE134025 were downloaded from the GEO database. DEGs were derived from samples of their colonic mucosa as well as healthy controls. These datasets consisted of endoscopically collected mucosal colonic biopsies emanating from ulcerative colitis patients and healthy volunteers. The GSE13367 model has been subjected to gene expression analysis using the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. The GSE13367 dataset consisted of 54 mucosal colonic biopsy samples in which 34 samples belonged to ulcerative colitis patients and 20 samples were taken from healthy controls. Gene expression analysis in the case of GSE134025 dataset was specifically performed with the help of GPL6947 Illumina Human HT-12 V3.0 expression bead chips. The GSE134025 dataset focused on the expression of genes in six samples, with three from ulcerative colitis patients and three from healthy subjects.

To identify DEGs, the variably expressed genes in each of the two datasets (GSE13367 and GSE134025) were initially analyzed individually with the criteria of a Fold Change (FC) greater than 1

and a p-value less than 0.05. Subsequently, the overlapping DEGs were determined by combining the two datasets, and this analysis was facilitated using the venny tool. This comprehensive approach allowed for the identification of common DEGs across the two datasets, providing valuable insights into gene expression changes associated with ulcerative colitis [6].

Analysis of the Functional and Pathway Associated with the DEGs

This research used the Metascape website which provides a comprehensive platform that seamlessly combines various tools and resources for biological analysis. It offers functional enrichment analysis, interaction analysis, genetic annotation, and the ability to search for specific memberships within datasets. This integration encompasses over 40 different knowledge databases [7]. Notably, we also used the Kyoto Encyclopedia of Genes and Genomes (KEGG) which serves as a repository within Metascape for highlighting the broad impacts and functions of biological systems [8]. Additionally, Gene Ontology (GO) was used to identify high-quality functional gene annotations across biological processes (BP), molecular functions (MF), and cellular components.

In our analysis, we configured Metascape with specific screening criteria, including a minimum overlap threshold of three and a minimum enrichment threshold of 1.5 (with a significance level of $p < 0.01$). These settings were applied to identify and elucidate the functions of Differentially Expressed Genes (DEGs).

PPI And Hub Genes Identification

The PPI network for the 197 DEGs was constructed, and 0.4 was the minimum required interaction score through the STRING database. Meaning that the PPI network active interaction sources were detected through text mining, co-expression, neighborhood, gene fusion, experiments, and databases among others. The Cytohubba ranking algorithm was applied in determining the hub genes of the 197 DEGs. Using the MCC algorithm and the cytoHubba Cytoscape plug-in, the top 5 hub genes were then identified. The functions of hub genes were predicted using Metascape with the screening settings of Min overlap of three and Minimum Enrichment of 1.5. which are Statistics considered significant at $p \geq 0.01$.

RESULT AND DISCUSSION

DEGs Identification

To refine the gene list, common genes from both datasets were filtered, and their representation was visualized using Venn diagrams (refer to Figure 1). 21.956 genes were found to be differentially expressed (DEGs). 9.990 DEGs were identified in GSE13367 meanwhile 18.487 DEGs were identified in GSE134025. Among these DEGs, 6521 were found to be differentially expressed, based on certain criteria $P<0.05$ and $logFC \geq 1$, we found 197 genes upregulated and downregulated significantly with 76 genes showing up-regulated and 121 genes showing down-regulated. Figure 2 illustrates the patterns of DEG gene expression in the two datasets, each containing two sets of sample data. It's noted that the majority of the up and downregulated genes were identified in GSE13367 (Figure 2a).

GO and KEGG Enrichment of the 197 DEGs

As per the Gene Ontology (GO) biological functional analysis, specifically depicted in Figure 3a, the 76 up-regulated genes exhibited significant enrichment in several key areas. In terms of biological processes, these genes were primarily associated with the regulation of antimicrobial humoral immune response mediated by antimicrobial peptides. In the realm of molecular functions, they were notably linked to peptidoglycan binding. Additionally, concerning cellular components, the up-regulated genes were enriched in the NADPH oxidase complex. Furthermore, the KEGG pathway analysis unveiled the predominant pathways associated with these 76 up-regulated genes. Notably, they were abundant in pathways related to viral protein interaction with cytokines and cytokine receptors, as well as pathways associated with amoebiasis. On the other hand, the 121 down-regulated genes exhibited distinct patterns of enrichment according to GO analysis (depicted in Figure 3b). These genes were prominently associated with functions related to transmitter-gated ion channel activity, inorganic anion

transmembrane transport, and plasma membrane integration, indicating a potential impact on cellular processes and membrane functions. Lastly, the KEGG analysis highlighted the primary functions associated with the 121 down-regulated genes. These genes were notably involved in the production and breakdown of keton substarnces indicating alterations in metabolic pathways associated with their downregulation.

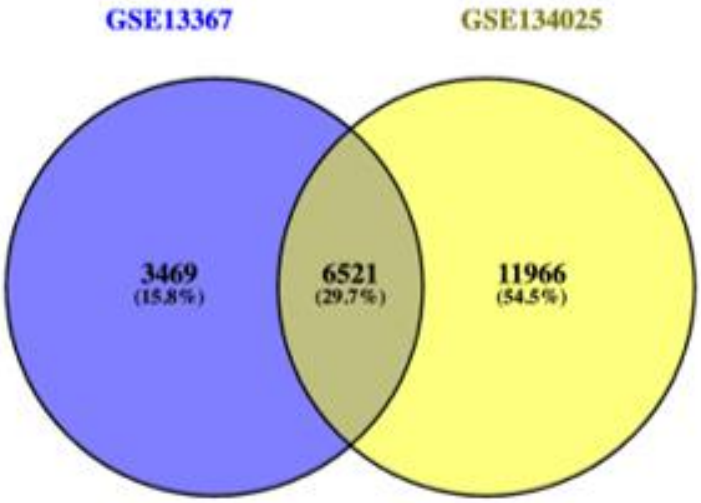
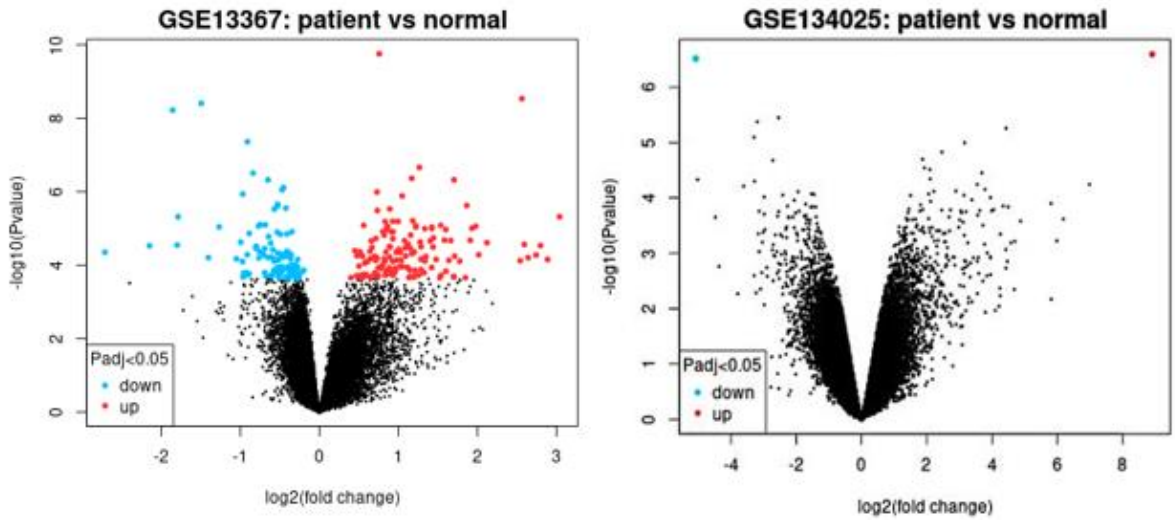
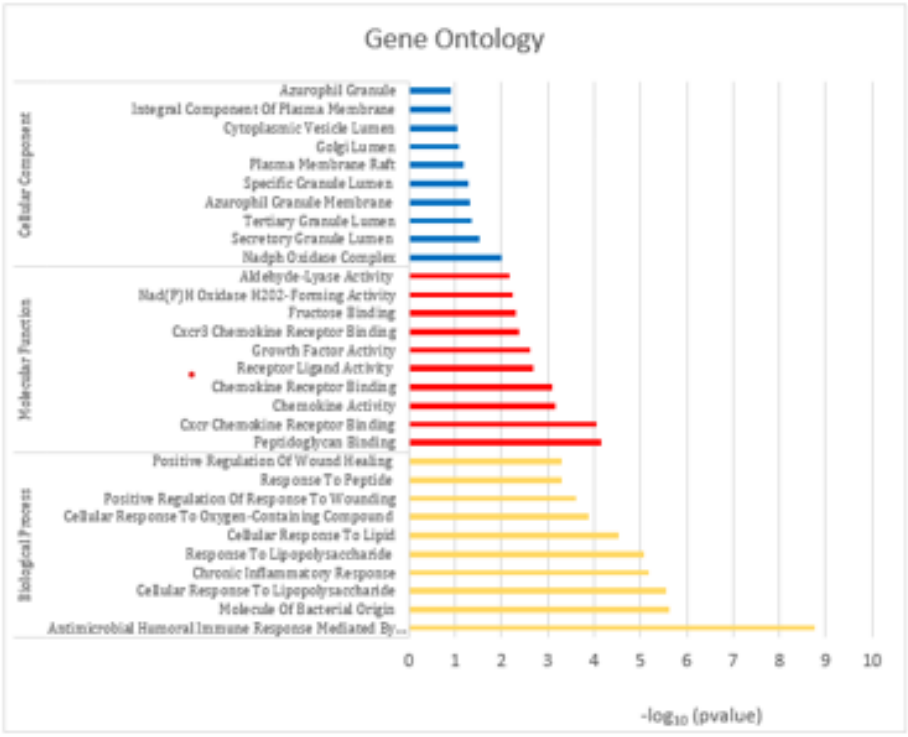


Figure 1. Venn diagram were used to visually represent the overlap of differentially expressed genes (DEGs) between the two Gene Expression Omnibus (GEO) datasets



(a) (b)

Figure 2. The volcano plots were drawn to present the differentially expressed genes for UC over control comparison. The DEGs from GSE13367 and GSE134025 datasets have been presented individually in (a-b). These plots depict the down-regulated genes with the blue points, the similarly up-regulated genes are indicated by red points and black points indicate the same expressed genes that showed no significant differences in expression



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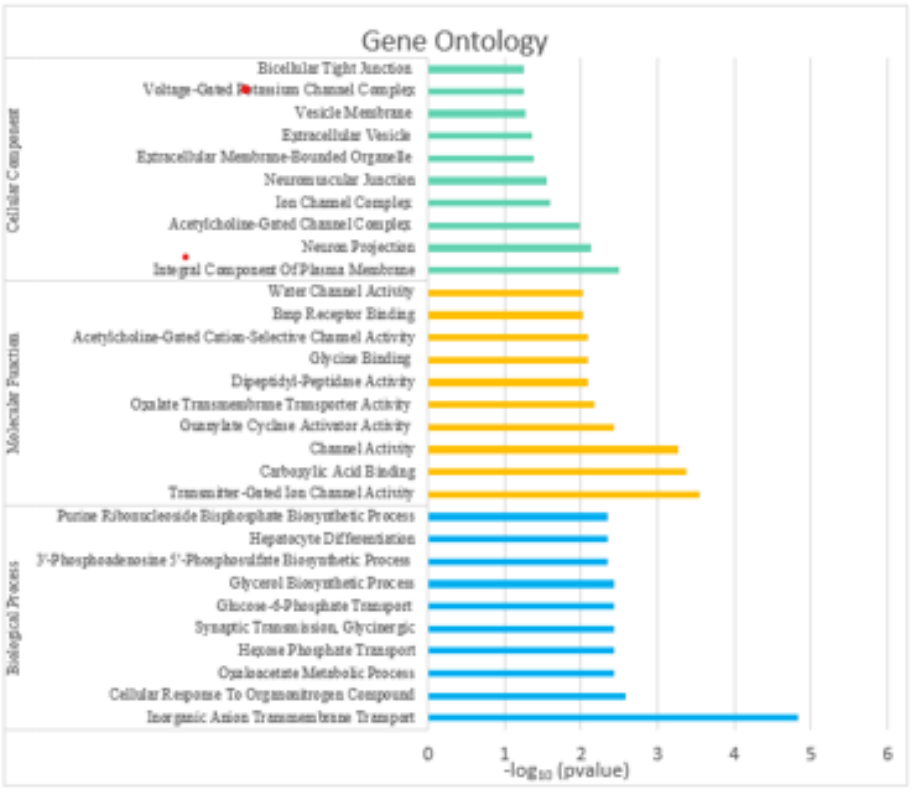


(b)

Figure 3. Analysis of functional enrichment in DEGs. The bar graphs illustrate the top 10 results of the up-regulated gene (a) and down-regulated gene enrichment analysis (b)

PPI Hub Genes Identification

The Protein-Protein Interaction (PPI) was done for network analysis of Differentially Expressed Genes (DEGs) using the STRING database, and the results are as shown in Figure 4a and 4b. The 76 up-regulated DEGs-formed PPI network contained 75 nodes and 190 edges, resulting in an average node degree of 5.07 and local clustering coefficient of 0.443. Taken together, these metrics point to a statistically significant enrichment in Protein-Protein Interactions (PPIs) amongst these up-regulated genes, with a p-value of 1.0e-16.

On the other, PPI network with 121 DEGs down-regulated compromised of 114 nodes with an average node degree of 1.11 and local clustering value of 0.258 in 63 edges. This network, like the up-regulated genes, also displayed a highly enriched PPI interaction with a p-value of 3.22e-09. Notably, the top five genes within the PPI network were identified using the MCC algorithm and the cytoHubba Cytoscape plug-in (Figure 5). These genes include CXCL11, CXCR4, CXCL13, CXCL1, and MMP3, suggesting their potential importance in the context of the analyzed DEGs and their associated biological processes.

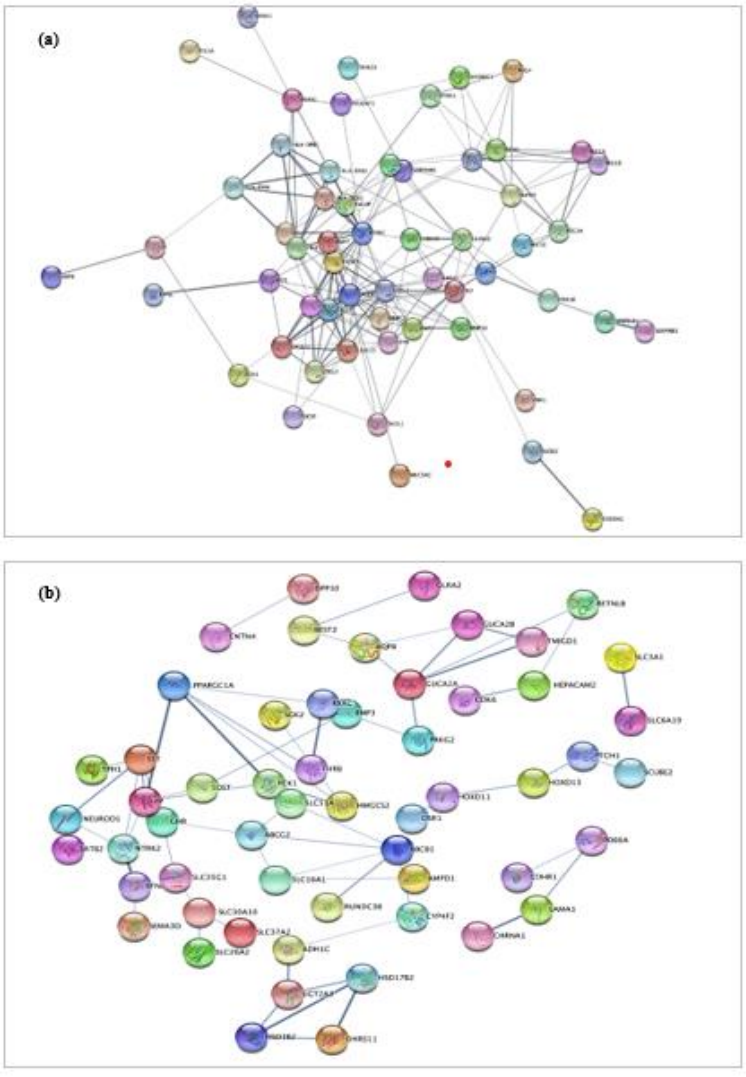


Figure 4. Cytoscape was used to create networks illustrating the protein-protein interactions (PPI) for the 76 up-regulated genes (a) and the 121 down-regulated genes (b). These networks visually portray the relationships and interactions among genes, where each gene is represented as a node. The connections or links between genes are illustrated as edges, and the size and color of each gene node indicate the extent of its interaction within the network

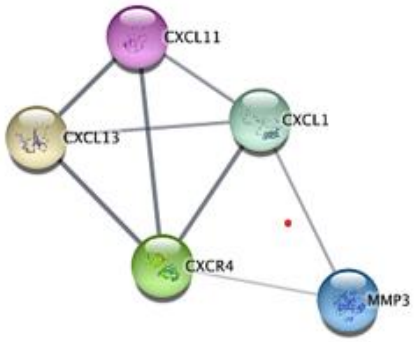


Figure 5. Cytoscape protein-protein interaction (PPI) networks were generated for the five identified hub genes through the MCC algorithm implemented in cytoHubba

Analysis of Hub Genes

The hub genes were subjected to functional enrichment analysis, yielding the following outcomes (as shown in Figure 6): Based on biological processes, the five hub genes were predominantly involved in the positive regulation of cell-cell adhesion mediated by integrin and the signaling pathways of chemokine ligand 12. Regarding molecular functions, they were notably associated with CXCR chemokine receptor binding, suggesting their role in binding to CXCR chemokine receptors. In terms of cellular components, the hub genes exhibited enrichment in the tertiary granule lumen, highlighting their presence in specific cellular compartments. Furthermore, when examining KEGG pathways, these five hub genes were predominantly enriched in pathways related to viral protein interaction with cytokines and cytokine receptors, suggesting their involvement in signaling pathways associated with viral interactions. These findings collectively provide insights into the functional roles of the identified hub genes within the context of the analyzed DEGs and their potential contributions to inflammatory and immune-related processes.

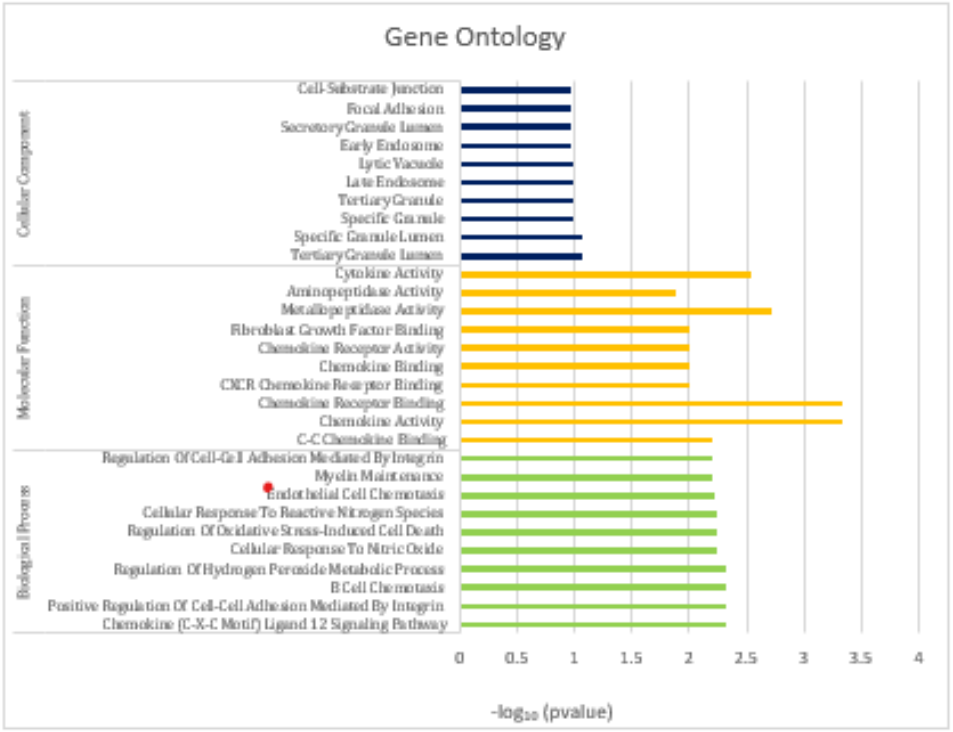


Figure 6. Evaluation of the functional enrichment of the five hub genes

Ulcerative colitis is a prevalent genetic inflammatory disorder of the colon characterized by symptoms such as bleeding, extensive fragility, and erosions on the colonic wall. This form of inflammatory bowel disease (IBD) is the most common worldwide, distinguished by its specific impact on the mucosa and submucosa of the colon. Typically, the disease initiates in the rectum and progresses inward [9]. Ulcerative colitis ranks among the most frequently occurring IBDs, with an incidence rate of twenty cases per 100,000 individuals in the United States. Both males and females are affected in nearly equal numbers [10]. Its prevalence is higher in populations in North America and Northern Europe compared to those in Asia. Given that gene expression patterns are frequently utilized to investigate ulcerative colitis, bioinformatics analysis serves as a valuable tool to uncover the pathophysiological mechanisms underlying UC [11].

In total, 197 differentially expressed genes (DEGs) were identified in this study among which 121 down-regulated genes and 76 up-regulated genes. From the Gene Ontology (GO) analysis, these DEGs were functionally categorized mainly to be associated with antimicrobial humoral immune response mediated through regulation of antimicrobial peptides. Additionally, they were linked to biological processes such as inorganic anion transmembrane transport. In terms of molecular function, the DEGs were associated with peptidoglycan binding and transmitter-gated ion channel activity. Regarding cellular components, they were enriched in the NADPH oxidase complex and integral components of the plasma membrane.

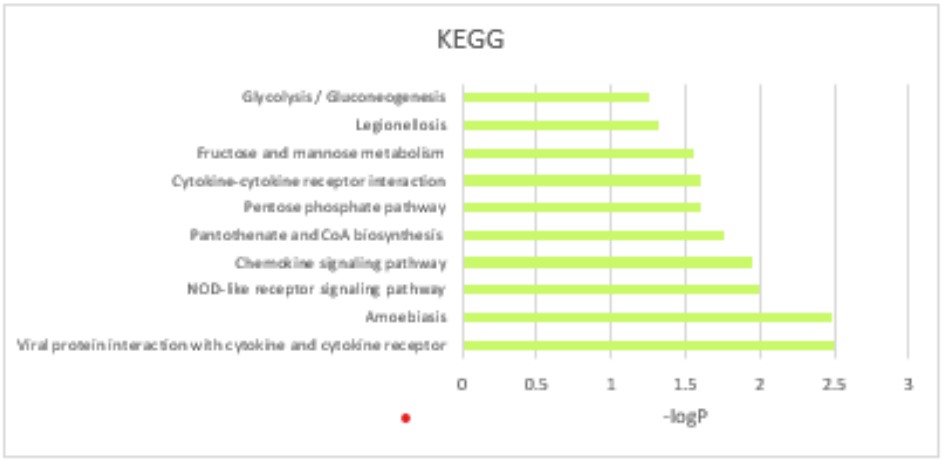
In addition, the KEGG pathway analysis showed that the DEGs that were upregulated were notably enriched in pathways associated with the interaction of viral proteins with cytokines and cytokine receptors, as well as in pathways related to amoebiasis. This aligns with the observation that the downregulated genes had extensive involvement in the synthesis and degradation of ketone bodies, as corroborated by KEGG pathway analysis. The both regulated DEGs had a very extensive involvement of glycolysis or gluconeogenesis (as demenosterated in Figure 7). Overall, this study sheds light on the molecular mechanisms underlying ulcerative colitis, providing valuable insights into the regulation of immune responses and metabolic pathways associated with this condition.

Among the 121 genes in the Protein-Protein Interaction (PPI) network of Differentially Expressed Genes (DEGs), five genes (CXCL11, CXCR4, CXCL13, CXCL1, MMP3) displayed a notable level of interaction, indicating their centrality in the network. In patients with ulcerative colitis (UC), all five of these hub genes exhibited increased expression levels. According to Gene Ontology (GO) analysis, these five genes were particularly enriched in the tertiary granule lumen as a cellular component, CXCR chemokine receptor binding as a molecular function, and the enhancement of cell-cell adhesion mediated by integrin as a biological process. Furthermore, when examining their involvement in signaling pathways, the five hub genes were predominantly associated with the TNF signaling pathway, viral protein interaction with cytokines and cytokine receptors, as demonstrated in KEGG pathway analysis (as demenosterated in Figure 8).

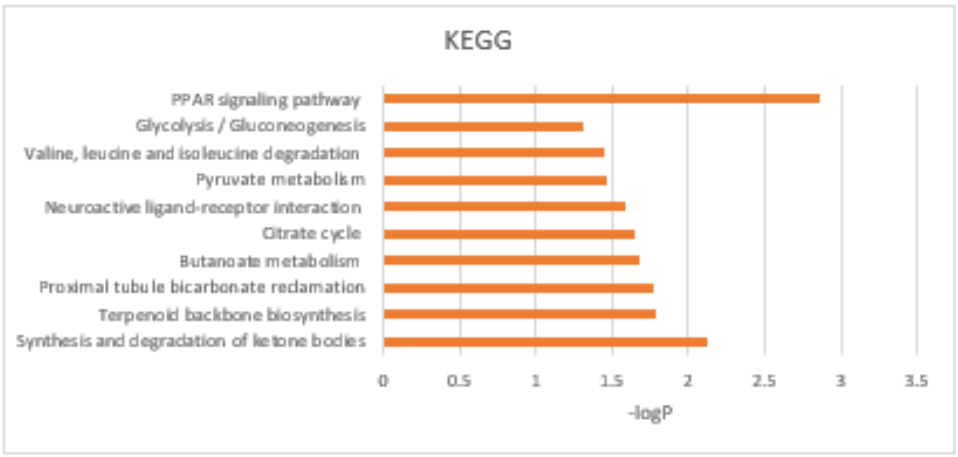
Ulcerative colitis (UC) is the idiopathic and recurrent inflammation of the mucosa of the intestines while its exact etiology is yet to be known. It usually initiates with the rectum and may go on to affect the entire colon. This engages multifaceted and dynamic web of cells and cytokines adjusting the immune response and inflammatory cascade within UC pathogenesis. Cytokine receptors on cell surfaces play a crucial role by binding precisely to cytokines and transmitting their signals, allowing cells to respond to signals from nearby or distant locations in the body [12]. The DEGs and hub genes identified in this study potentially contribute to the development of UC by participating in these intricate mechanisms, shedding light on the molecular pathways underlying the disease and providing a basis for further investigation into therapeutic interventions.

Table 1 presents the hub gene symbols, acronyms, and their corresponding functions. CXC motif chemokine ligand 1 (CXCL1) belongs to one of the four subfamilies comprising around 50 chemotactic cytokines [18]. This subgroup is distinguishable by the presence of a pair of disulfide bridges formed through the homologous CXC motif. CXCL1 plays a crucial role in the development of inflammatory bowel disease. Both ulcerative colitis and Crohn's disease patients have been found to have elevated blood levels of CXCL1, with ulcerative colitis patients having higher levels [16]. CXCL1 shows promise as a potential biomarker for ulcerative colitis and has been identified as a hub gene in ulcerative colitis through gene expression analyses. In the context of inflammatory bowel disease, CXCL1 contributes to

the chemotaxis of neutrophils and their infiltration into sites of inflammatory responses. It's important to note that in inflammatory bowel disease, CXCL1 is not the sole chemoattractant for neutrophils [19].



(a)



(b)

Figure 7. KEGG pathway analysis of functional enrichment in DEGs. The bar graphs illustrate the top 10 results of the up-regulated gene (a) and down-regulated gene enrichment analysis (b)

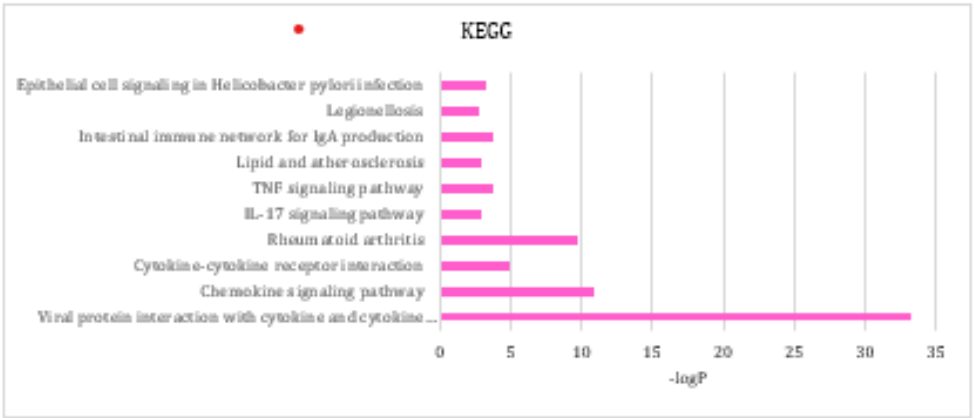


Figure 8. KEGG pathway analysis of 5 hub genes. The bar chart depict the top ten findings for the five hub genes

Table 1. Five hub genes and their functions

Gene symbol	Description	Function
CXCL11	chemokine ligand 11	A well-known controller of T-cell entry into tumors is CXCL11 [13].
CXCR4	C-X-C chemokine receptor type 4	CXCR4 produces a protein that penetrates outer cell layers, including those in the brain, spinal cord, and white blood cells [14].
CXCL13	C-X-C Motif Chemokine Ligand 13	(CXC chemokine ligand 13) has been proved to be generated persistently by stromal cells in lymphoid follicles of human lymph nodes and It serves as a potent attractant for naïve B cells in laboratory settings. This chemokine was also named B lymphocyte chemoattractant (BLC) and was initially discovered in mice [15].
CXCL1	C-X-C Motif Chemokine Ligand 1	Melanomagrowth-stimulating activity/growth-regulated protein, or CXCL1, is chemokine that is crucial for inflammation, angiogenesis, carcinogenesis, and wound healing [16].
MMP3	Stromelysin-1or as Transin-1	Extracellular matrix (ECM) components including matrix proteins, growth factors, proteases,surface receptors, and adhesion molecules can all be broken down by MMP-3 [17].

On the other hand, CXCL13 was initially identified in B cell follicle stromal cells. CXCL13 plays a pivotal role in the pathogenesis of various inflammatory diseases, including autoimmunity. It is constitutively expressed in secondary lymphoid tissues and promotes lymphoid neogenesis when expressed [20]. The presence of CXCL13 in human inflammatory diseases such as ulcerative colitis, chronic gastritis caused by Helicobacter pylori, and chronic inflammation associated with human lymphoid neogenesis suggests a potentially harmful role for CXCL13 [21].

In ulcerative colitis, the ELR-chemokine CXCL13 exhibits increased CXCL13 mRNA expression in intestinal tissues and interacts with CXCR5 as its receptor. These molecular insights provide valuable information about the involvement of CXCL1 and CXCL13 in the pathogenesis of ulcerative colitis and other inflammatory conditions [22].

Chemokines, essential for various biological processes including growth and homeostasis, exert significant effects on central nervous system cells and endothelial cells involved in both angiogenesis and angiostasis. Among these chemokines, CXCL11 stands out as the most potent CXCR3 agonist based on chemotaxis experiments. CXCL11 primarily induces T cell transepithelial migration and leads to receptor down-regulation[23]. Interestingly, CXCL10 and CXCL11 practiced enormous mRNA valorisation in of ulcerative colitis (UC) as well as colorectal cancer (CRC). Moreover, miR-34a-5p and miR-203a-5p appear to be potential regulators' miRNAs for CXCL10 and CXCL11. In the context of UC, interactions between CXCL10 and CXCL11 and cytokine receptors can activate the JAK-STAT signaling cascade. Furthermore, it has been observed that in both UC and CRC, CXCL10 and CXCL11 are positively correlated with the tissue infiltration of proinflammatory M1 macrophages [24].

CXCR4 (Chemokine (C-X-C motif) Receptor 4 represents a specific subclass of G protein-coupled receptors (GPCRs) that bears resemblance to an amino acid rhodopsin. Notebly It was shown that in the peripheral blood of UC patients the number of immature plasma cells increases significantly versus healthy donors and further reveals severe CXCR4 overexpression [25].

A study by Mina T. shows that these particular IgG plasma cells mediate the exacerbation of mucosal inflammation, functioning as one of the key elements in the pathogenesis of UC. This is through CXCR4 infiltration in inflamed mucosa to initiate "pathogenic" intestinal CD14 macrophages by means of IgG-ICFrC signaling. These findings provide valuable insights into the complex interplay of chemokines, receptors, and immune cells in the context of ulcerative colitis pathogenesis [26].

The extracellular matrix (ECM) is an important structural component of tissues normally degraded by proteins of the matrix metalloproteinase (MMP) family. MMPs have two roles to play firstly that in pathological circumstances as in arthritis and metastasis, and those in beneficial physiological activities like embryonic development, reproduction, and remodeling of tissues [27]. Many MMPs are initially released as inactive proproteins, which can be activated by extracellular proteinases. In the context of mucosal degeneration induced by the pokeweed mitogen, pharmaceutical inhibition of MMP-3 has been shown to effectively halt this degenerative process [28]. This highlights the significance of MMPs in tissue homeostasis and their potential as therapeutic targets in certain pathological conditions.

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AUTHOR CONTRIBUTIONS

Concept: O.A.O.A., L.E., F.F.; Design: O.A.O.A., L.E.; Supervision: F.F., B.H.; Resources: F.F., B.H.; Materials: L.E., F.F., B.H.; Data Collection and/or Processing: O.A.O.A., L.E.; Analysis and/or Interpretation: O.A.O.A., L.E.; Literature Search: O.A.O.A., L.E.; Writing: O.A.O.A.; Critical Reviews: L.E., F.E., B.H.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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