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Original research

In silico prediction of differentially expressed genes and functionally grouped networks in patients with inflamed pulp for screening pulpitis biomarkers

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Purpose
Pulpitis is one of the most common oral inflammatory diseases. There are many limitations in the traditional methods of diagnosing pulpitis. By replacing new diagnostic ways based on biomarkers, it is possible to quickly and accurately identify this disease. Biological indicators have greatly helped not only in the screening of infectious diseases but also in early and appropriate treatment. In this research, differentially expressed genes (DEGs) related to pulpitis were analyzed, and prognostic biomarkers were introduced.

Materials and Methods
In this *in silico* study, we applied the GSE77459 dataset as the gene expression profile of pulpitis. Web tool, GEO2R was used to separate up-regulated and down-regulated DEGs. |logFC|>2 and adjusted p-value < 0.05 was set as the cut-off criterion. For the pathway enrichment study of obtained genes, EnrichR was implemented. After constructing a protein-protein interaction (PPI) network, hub genes that are involved in pulpitis were selected. Finally, functionally grouped networks by ClueGO software (v2.5.10) were generated.

Results
GEO2R analysis of the GSE77459 dataset showed 672 up-regulated genes and 239 down-regulated genes with GB_ACC code. Based on Cytoscape results, the 15 top hubba nodes were ranked including PTPRC, ITGAM, CCL2, ICAM1, MMP9, CXCL8, TLR2, CD86, CXCR4, IL1A, CD44, CCL3, ITGAX, CXCL10, and CCR7. Functionally grouped networks determined that these genes were mainly enriched in chemokine-mediated signaling pathway, morphogenesis of endothelium, and neuroinflammatory response

Conclusion
In our research, 15 genes were introduced as diagnostic biomarkers in pulpitis and their functionally grouped networks were constructed. However, the obtained results need to be validated using in vitro and in vivo methods.

Keywords: *Dental pulp, inflammation, gene ontology, DEGs, PPI, Pulpitis*

Introduction

Under the dentin is the innermost and vital part of the tooth called the dental pulp (1). This jelly-like center is a mass of connective tissue and the presence of proteins such as albumin, transferrin, tenascin, and other proteoglycans is necessary for its proper function (1, 2). Large nerve trunks and blood vessels in the central area of the pulp play a major role in signaling processes and nerve message transmission. In addition, dentin formation, dentin nutrition, and tooth defense are important functions related to the dental pulp (1-3).

Delay in the treatment of tooth decay leads to damage in the hard tissue of the tooth and various stimuli cause pathological changes in the

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dental pulp and surrounding periapical tissues which eventually leads to pulpitis. As a result of uncontrolled inflammation caused by invading bacteria, irreversible pulpitis occurs (4). Based on the degree of pulp inflammation and the importance of tooth preservation, dentists choose different strategies for the treatment of pulpitis such as covering the pulp, staged decay drilling, and pulpotomy (5, 6). To preserve the infected tooth, it is very important to quickly recognize and prescribe appropriate treatment for patients. Traditional methods of diagnosing pulpitis are based on clinical findings and the results of X-rays and pulp vital tests, which have limitations and need to be refined (7).

One of the interesting topics in medical science is the identification of molecular biomarkers for early diagnosis of diseases (8, 9). Diagnostic biomarkers are important in drug selection, treatment response, prevention, and other aspects of biomedicine (10). The use of computer-based methods with high throughput has accelerated the process of analysis. One of the ways to identify molecular biomarkers is using microarray technology and the study of differentially expressed genes (DEGs) between two states (healthy and diseased states) (11, 12). To draw the interaction network of DEGs that reflects specific conditions, and physical interactions on a wide scale, a protein-protein interaction network (PPIN) is used. Cytoscape software constructs a network of all the differentially expressed genes along with the determination of degree, betweenness, and closeness. The number of links of a given node determines its degree centrality. Nodes with a higher degree are considered hub genes (8, 13). To investigate the biological pathways related to these hub genes, functionally grouped network analysis in ClueGO software can be used (14).

There is a lot of evidence that shows the important role of differentially expressed mRNAs (DEMs), miRNAs (DEMIs), and lncRNAs (DELs) in a variety of cellular and pathological processes along with pulpitis. With the RT-qPCR technique, the difference in the expression levels of many mRNAs such as, HMOX1, LOX, ACTG1, STAT3, GNB5 has been proven (15). Previous studies have shown that some miRNAs for example miR-155, miR-21, miR-142, miR-223, miR-486, miR-675 were significantly upregulated in patients with inflamed pulp (16). Further, Huang *et al.* (17) showed that 752 lncRNAs were significantly altered in inflamed pulp samples compared to normal pulp samples, they reported 338 overexpressed lncRNAs and 414 down-regulated lncRNAs. However, detailed research on functionally grouped networks based on key differentially expressed genes seems necessary to study key pathways related to pulpitis. In our research, due to the importance of discovering critical pathways related to pulpitis and an improved method in the early detection of this disease, first key differentially expressed genes related to pulpitis were introduced, and then, functionally grouped network for detected biomarkers was constructed.

Materials and Methods

Microarray data extraction

The workflow chart of data processing and analysis in this study is shown in Figure 1. Microarray data related to patients with inflamed pulp was extracted from a public database called Gene Expression Omnibus (GEO). Based on the keywords including pulpitis, Homo sapiens in the type of organism part, series in the entry type part, and expression profiling by an array in the study type section, searches were limited.

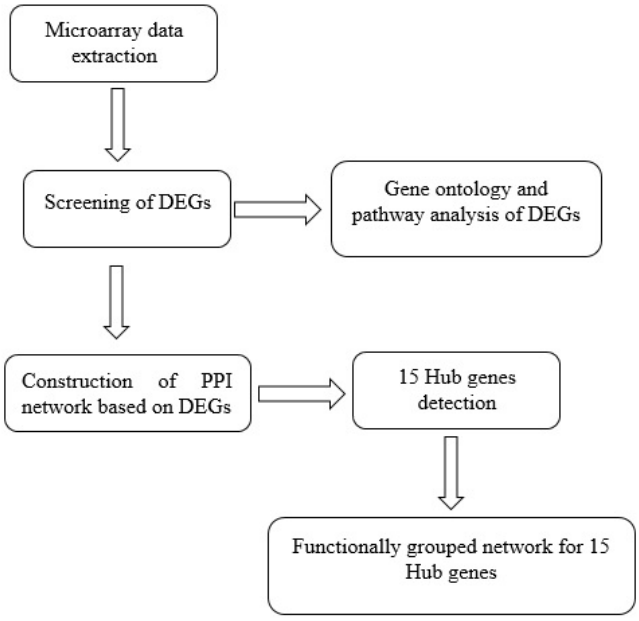

Figure 1. *Flowchart of data processing and analysis.*

Screening of DEGs

Screening of DEGs between samples from inflamed pulps, and samples from normal pulps were analyzed via GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r). The tool system of GEO2R works using the R language. The normal distribution of data in box plots of this dataset was checked and then, to estimate differentially expressed genes $|logFC|>2$ and adjusted p-value < 0.05 were regarded as statistically significant.

Gene ontology and KEGG pathway analysis of DEGs

For a more in-depth understanding of the obtained DEGs, Gene ontology and KEGG pathway analysis were carried out by EnrichR.This analysis tool is useful for predicting the most significantly enriched pathways of DEGs in biological processes, molecular functions, cellular components, and human phenotype Ontology.

Construction of PPI network

For PPI network construction, based on the genes that had a significant increase and decrease in expression, STRING v9. 1 software in https://string-db.org was used. After selection Homo sapiens in organism type search, DEGs were inserted as multiple proteins. Finally, protein-protein interaction network with a high confidence score ≥ 0.7 was formed. The output file was imported into Cytoscape (v3.9.1) software for more analysis.

Hub genes detection

CytoHubba with free access is a functional section in the menu bar of Cytoscape software that is very helpful in dis-

covering hub protein in protein-protein interaction networks. The basis for identifying key genes in the CytoHubba plugin is the degree, maximum neighborhood component, and other topological analysis parameters. In this research, 15 hub nodes were obtained using this plugin.

Functionally grouped networks for Hub genes

The key genes obtained from the previous step were considered for functionally grouped network studies. The ClueGO software (v2.5.10) was used for this target. This functional software provides a network for hub genes using various original and reliable sources. To deeply investigate the relationship between these genes and pulpitis formation, the pathways with p-values less than 0.05 were filtered.

Results

Microarray data extraction and Screening of DEGs

In our study, accession number GSE77459 was selected as the gene expression profile dataset with inflamed pulp samples for further analysis. GSE77459 which has been studied by the GPL17692 platform (Affymetrix Human Gene 2.1 ST Array) contains 6 samples of irreversible inflamed pulps and 6 samples of normal pulps. Differentially expressed genes that were detected from GSE77459 by GEO2R include 672 upregulated genes and 239 downregulated genes with GB_ACC code. The box plot and volcano plot of this dataset were presented in Figures 2A and 2B.

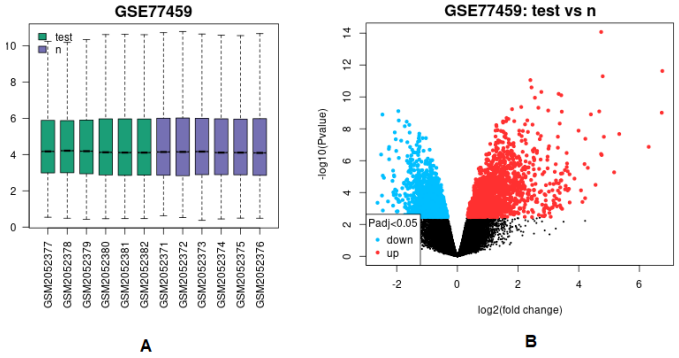

Figure 2. *A: The box plot of GSE77459; B: Volcano plot of GSE77459.*

Gene ontology and pathway analysis of DEGs

Based on GO biological process, the differentially expressed genes were enriched in Cytokine-Mediated Signaling Pathway (GO:0019221), Inflammatory Response (GO:0006954), Neutrophil Chemotaxis (GO:0030593), Granulocyte Chemotaxis (GO:0071621), Neutrophil Migration (GO:1990266), Cellular Response To Lipopolysaccharide (GO:0071222). Kyoto encyclopedia of genes and genomes (KEGG) analysis showed, Cytokine-cytokine receptor interaction, Viral protein interaction with cytokine and cytokine receptor, Chemokine signaling pathway, TNF signaling pathway, Staphylococcus aureus infection, and Cell adhesion molecules are major pathways in which DEGs are involved. The P-value, adjusted P-value, odds Ratio, and Combined score of each pathways are shown in Table 1 and 2.

Construction of PPI network and hub genes detection

The protein network generated by STRING v9. 1 was visualized with Cytoscape software. This network consists of 564 nodes and 6704 edges (Figure 3). After calculating the score of each node by the CytoHubba plugin, 15 hub genes were screened. The 15 top hubba nodes were ranked based on the degree including PTPRC, ITGAM, CCL2, ICAM1, MMP9, CXCL8, TLR2, CD86, CXCR4, IL1A, CD44, CCL3, ITGAX, CXCL10, and CCR7. In this selection, the criterion above 102 was considered. The gene symbols, scores, and ranks of each gene are shown in Table 3.

Functionally grouped network for Hub genes

Functional annotation of the hub genes is shown in Figure 4. hub genes were analyzed by ClueGO software (v2.5.10). The output file was a network with 3 groups (PV < 0.05). chemokine-mediated signaling pathway, morphogenesis of endothelium, and neuroinflammatory response were 3 major groups in this network.

Discussion

The presence of microorganisms in the pulp space causes pulpitis (18). The progress of infection in the dental pulp stimulates the pain receptors causing a pain response which is the main reason for emergency visits to dentists (19). Early diagnosis of inflamed pulp is very important to provide appropriate treatment. Biological markers are measurable parameters and are important indicators for checking normal conditions. Like any other inflammation, pulpitis is accompanied by changes in biological molecules (20-22). These biological differences between healthy and inflamed dental pulp can be the basis of rapid diagnosis. Due to the lack of a reliable reference standard in the diagnosis of pulpitis, the identification of new biomarkers, along with previous methods, can be a suitable strategy for the diagnosis of inflamed pulp before reaching the stage of experiencing progressive and devastating pain (22). In this study, differentially expressed genes (DEGs), PPI networks, and hub genes were investigated by analyzing and comparing the expression matrix of genes in the inflamed pulp. Finally, prognostic biomarkers and functionally grouped networks were introduced.

In our study, in order to compare differentially expressed genes between healthy and inflamed dental pulp, the gene expression profile dataset with accession number GSE77459 was analyzed and 672 upregulated genes and 239 downregulated genes were identified. Among differentially expressed genes, The 15 top hubba nodes were detected as diagnostic biomarkers including PTPRC, ITGAM, CCL2, ICAM1, MMP9, CXCL8, TLR2, CD86, CXCR4, IL1A, CD44, CCL3, ITGAX, CXCL10, and CCR7.

The activity of most obtained genes is related to inflammatory responses. Tyrosine phosphatase receptor type C (PTPRC) is a transmembrane glycoprotein that has important roles in the regulatory processes of cell growth, differentiation, mitosis, and transformation. PTPRC is also known as CD45 (23, 24). An increase in CD45 indicates activation of one or more inflammatory conditions (25). Integrin alpha

Table 1. *Gene ontology results of DEGs.*

Term	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
Cytokine-Mediated Signaling Pathway (GO:0019221)	5.89E-34	1.51E-30	11.64114	890.7257	CNTFR;CXCL6;CSF3;PLVAP;CXCL9; SPI1;CSF3R;CXCL8;CXCL1;CXCL13; CXCL3;CXCL2;CXCL5;OASL;GHR;IL6R; PF4V1;CCR2;FCER1G;SYK;IL18;OSMR; TNFRSF1B;EREG;HCK;IL1A;CEACAM1; AIM2;KIT;IL3RA;BIRC3;CSF2RB;IL2RG; CSF2RA;CCL4;STAT4;CCL3;CCL2; CD300LF;LYN;EGR1;IL33;CCL23; CCL21;CCL20;OSM;LILRB1;PPBP; CXCL10;CXCL11;LEP;PTPN6;PF4
Inflammatory Response (GO:0006954)	1.96E-28	2.51E-25	10.68248	681.5376	CXCL6;CXCL9;CXCL8;C5AR1; FPR1; CXCR4;PIK3CD;FPR3;CXCL1; HPR;F11R; CXCL13;CXCL3;ITGAL;CXCL2; CXCL5;CCL4;CCL3;CCL2;CCR7; PF4V1;CMKLR1;CCR2;CD96; CCL23;CCL21;CCL20;SLC11A1; NFAM1;IL18;CYBB;PPBP;FOS; TACR1;SELE;IL1A;CXCL10; CXCL11;KIT;CHI3L1;ACKR1; S100A9;CD44;S100A8;TLR2;PF4
Neutrophil Chemotaxis (GO:0030593)	2.81E-27	2.40E-24	28.47076	1740.634	CXCL6;CXCL9;CXCL8;PIK3CD; CXCL1;CXCL13;CXCL3;CXCL2; TREM1;CXCL5;CXCR1;CCL4; CCL3;CCL2;PF4V1;EDN1;CCL23; FCER1G;SYK;CCL21;CCL20;PPBP; CXCL10;CXCL11;JAML;S100A9; S100A8;PF4
Granulocyte Chemotaxis (GO:0071621)	1.15E-26	7.37E-24	26.56862	1586.856	CXCL6;CXCL9;CXCL8;PIK3CD; CXCL1;CXCL13;CXCL3;CXCL2; TREM1;CXCL5;CXCR1;CCL4; CCL3;CCL2;PF4V1;EDN1;CCL23; FCER1G;SYK;CCL21;CCL20;PPBP; CXCL10;CXCL11;JAML;S100A9; S100A8;PF4
Neutrophil Migration (GO:1990266)	6.72E-26	3.44E-23	24.39474	1413.957	CXCL6;CXCL9;CXCL8;PIK3CD;CXCL1;CXCL13;CXCL3; CXCL2;TREM1;CXCL5;CXCR1;CCL4;CCL3;CCL2;PF4V1; EDN1;CCL23;FCER1G;SYK;CCL21;CCL20;PPBP;CXCL10; CXCL11;JAML;S100A9;S100A8;PF4
Cellular Response To Lipopolysaccharide (GO:0071222)	4.09E-24	1.75E-21	14.94729	804.9457	CD86;CD274;CXCL6;CXCL9;CXCL8;CD80;SERPINE1; TNFAIP3;CXCL1;PTPN22;CXCL13;CXCL3;CXCL2;CXCL5; PLCG2;CCL3;SIRPA;CCL2;PF4V1;LYN;IL18;LILRB1; NR1D1;PPBP;TNFRSF1B;CXCL10;HCK;IL1A;CXCL11; CARD16;CD68;PF4

M (ITGAM) also known as CD11b, is expressed in myeloid and lymphoid cells. Expression of CD11b increase in monocytes in inflammatory response (26). C-C chemokine ligand 2 (CCL2) is produced in response to pro-inflammatory cytokines and increased in inflamed dental pulp (27). Among Glycosylated proteins on the surface of endothelial cells and immune cells that are up-regulated in inflamed tissue is intercellular adhesion molecule 1 (ICAM1) which binds to integrins (28, 29). Matrix metalloproteinase 9 (MMP9) is involved in the proteolysis of the extracellular matrix as well as the migration of leukocytes (30). Sharma *et al.* (31) reported that MMP-9 is a potential prognostic biomarker in patients with irreversible pulpitis. C-X-C Motif Chemokine Ligand 8 (CXCL8) acts as a primary cytokine in the inflammation site for neutrophil recruitment. In a study conducted by Karapanou *et al.* (32) the concentration of CXCL8 in gingival crevicular fluid (GCF) samples in pulpal inflammation was investigated and the increase of this inflammatory factor was confirmed (33). Gram-positive bacteria in the pulp area cause the activation and increase of Toll-Like Re-

Table 2. *KEGG pathway assessment of DEGs.*

Term	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
Cytokine-cytokine receptor interaction	5.85E-26	1.47E-23	8.588493	498.9977	CNTFR;CXCL6;IL1RN;CSF3;CXCL9;CSF3R;CXCL8; CXCR4;CSF2RB;CXCL1;CXCL13;CXCL3; IL2RG; CSF2RA;CXCL2;CXCL5;TNFSF13B;GHR; CXCR1;CXCR3;CCL4;TNFSF10;CCL3; TNFRSF17;CCL2;CCR7;IL6R;PF4V1; CCR2;IL33;CCL23;CCL21;CCL20;IL1R2; IL10RA;LIF;IL18;OSM;IL16;PPBP;OSMR; TNFRSF1B;IL1A;CXCL10;CXCL11;LEP;IL3RA;PF4
Viral protein interaction with cytokine and cytokine receptor	8.24E-25	1.04E-22	18.35683	1017.994	CXCL6; CXCL9;CXCL8;CXCR4;CXCL1;CXCL13;CXCL3;IL2RG; CXCL2;CXCL5;CXCR1;CXCR3;CCL4;TNFSF10;CCL3;CCL2;CCR7; IL6R;PF4V1;CCR2;CCL23;CCL21;CCL20;IL10RA;IL18;PPBP; TNFRSF1B;CXCL10;CXCL11;PF4
Chemokine signaling pathway	7.21E-22	4.54E-20	9.972527	485.4778	CXCL6;CXCL9;CXCL8;ADCY4;PIK3CD;CXCR4;CXCL1;ARRB2; CXCL13;CXCL3;CXCL2;CXCL5;PIK3R5;CXCR1;CXCR3;CCL4; PLCG2;RAC2;CCL3;CCL2;CCR7;PF4V1;CCR2;LYN;CCL23;CCL21; CCL20;PPBP;FGR;CXCL10;HCK;CXCL11;ELMO1;DOCK2; PLCB2;PF4
TNF signaling pathway	1.45E-18	7.29E-17	12.82573	526.8552	CXCL6;PIK3CD;TNFAIP3;CXCL1;CXCL3;PTGS2;CXCL2;CXCL5; ICAM1;SOCS3;CASP10;CCL2;JUNB;MAP2K6;EDN1;MLKL; RIPK3;CCL20;LIF;FOS;TNFRSF1B;SELE;MMP9;CXCL10; BCL3;BIRC3
Staphylococcus aureus infection	6.95E-16	2.50E-14	12.68297	442.6667	ITGAM;SELPLG;C5AR1;CFI;PTAFR;FPR1;FPR3;ITGAL;FCAR; ICAM1;C2;C4B;C3;C4A;HLA-DMA;HLA-DMB;FCGR2A; HLA-DRA;HLA-DOA;FCGR1A;HLA-DOB;HLA-DQA1
Cell adhesion molecules	2.05E-15	6.47E-14	9.02434	305.1966	CD86;CD274;ITGAM;SELPLG;CD80;NRXN1;ICAM2;F11R; ITGAL;ICAM1;SPN;CDH5;HLA-DMA;HLA-DMB;CTLA4; HLA-DOA;ICOS;HLA-DOB;HLA-DQA1;HLA-B;SELE;CLDN5; PTPRC;SELL;CNTN1;HLA-DRA

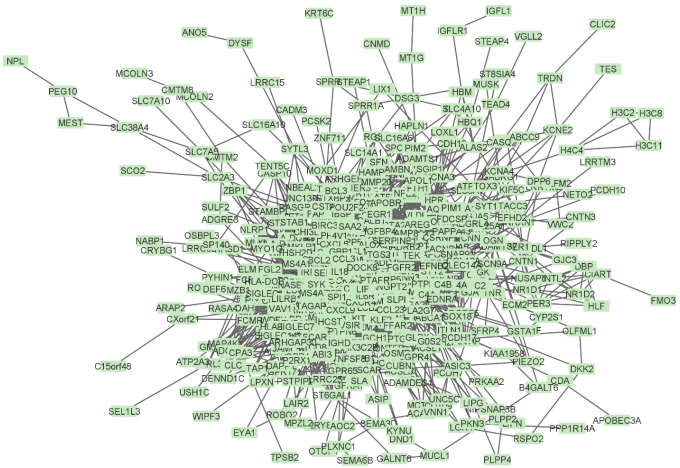

Figure 3. *Protein-protein interaction network of DEGs.*

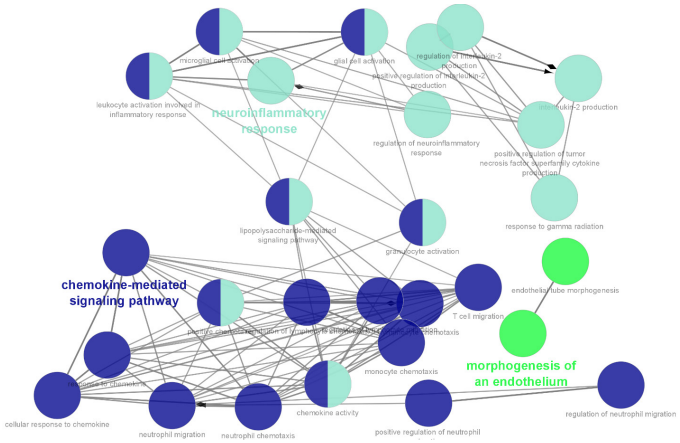

Figure 4. *Functionally grouped network for Hub genes.*

ceptor 2 (TLR2) expression in adult mice with infected pulp (34). Cluster of Differentiation 86 (CD86) is expressed by macrophages and B lymphocytes which play an essential role in the inflammation (35).

So far, many studies have been conducted on pulpitis biomarkers. Chen *et al.* (36) merged two datasets and reported hub nodes from the common genes of two profiles. In their study, 8 diagnostic biomarker candidates were reported which include PTPRC, CD86, CCL2, IL6, TLR8, MMP9, CXCL8, and ICAM1. In our research, the hub genes of one microarray gene expression dataset related to pulpitis were subjected to ClueGO software (v2.5.10) for functionally grouped network determination. Based on this network, our hub genes) PTPRC, ITGAM, CCL2, ICAM1, MMP9, CXCL8, TLR2, CD86, CXCR4, IL1A, CD44, CCL3, ITGAX, CXCL10, and CCR7 (were mainly enriched in the chemokine-mediated signaling pathway, morphogenesis of endothelium, and neuroinflammatory response. Detection of target proteins in new drug development is the major challenge. These hub genes can be useful not only in the screening of Pulp inflammation, they can provide some new ideas for further research such as therapeutic drug development for clinical application.

Table 3. *Ranks, Score, gene symbol of the top 15 hub genes that screened with CytoHubba plugin.*

ranks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Score	173	152	134	132	129	126	122	120	110	109	105	105	105	104	102
gene symbol	PTPRC	ITGAM	CCL2	ICAM1	MMP9	CXCL8	TLR2	CD86	CXCR4	IL1A	CD44	CCL3	ITGAX	CXCL10	CCR7
Description	protein tyrosine phosphatase receptor type C	Integrin Subunit Alpha M	C-C chemokine ligand 2	Intercellular Adhesion Molecule 1	Matrix Metallo peptidase 9	C-X-C Motif Chemokine Ligand 8	Toll Like Receptor 2	Cluster of Differentiation 86	C-X-C Motif Chemokine Receptor 4	Interleukin 1 alpha	Cluster of Differentiation 44	C-C Motif Chemokine Ligand 3	(Integrin Subunit Alpha X	C-X-C Motif Chemokine Ligand 10	C-C chemokine receptor type 7

Conclusion

In this study after analyzing the GSE77459 dataset in the GEO database, the 15 biomarker were detected. Functionally grouped networks show these genes were mainly enriched in pathways related to inflammatory responses. However, the obtained results need to be validated by in vitro and in vivo methods.

Türkçe özet: *Pulpitis biyobelirteçlerini taramak için iltihaplı pulpalı hastalarda diferansiyel olarak eksprese edilen genlerin ve fonksiyonel olarak gruplandırılmış ağların silico tahmini Amaç: Pulpitis en sık görülen oral inflamatuar hastalıklardan biridir. Pulpitis tanısının geleneksel yöntemlerinde birçok sınırlama vardır. Biyobelirteçlere dayalı yeni tanı yöntemlerinin değiştirilmesiyle bu hastalığın hızlı ve doğru bir şekilde tanımlanması mümkün olmaktadır. Biyolojik göstergeler sadece bulaşıcı hastalıkların taranmasında değil, aynı zamanda erken ve uygun tedavide de büyük ölçüde yardımcı olmuştur. Bu araştırmada pulpitis ile ilgili diferansiyel olarak eksprese edilen genler (DEG'ler) analiz edilmiş ve prognostik biyobelirteçler tanıtılmıştır. Gereç ve yöntem: Bu in silico çalışmasında, pulpitisin gen ekspresyon profili olarak GSE77459 veri setini uyguladık. Web aracı GEO2R, yukarı regüle edilmiş ve aşağı regüle edilmiş DEG'leri ayırmak için kullanıldı. |logFC|>2 ve düzeltilmiş p değeri < 0,05 kesme kriteri olarak belirlendi. Elde edilen genlerin yol zenginleştirme çalışması için EnrichR uygulandı. Bir protein-protein etkileşimi (PPI) ağı oluşturulduktan sonra pulpitiste rol oynayan merkez genler seçildi. Son olarak, ClueGO yazılımı (v2.5.10) tarafından işlevsel olarak gruplandırılmış ağlar oluşturuldu. Bulgular: GSE77459 veri setinin GEO2R analizi, GB_ACC kodlu 672 yukarı regüle edilmiş gen ve 239 aşağı regüle edilmiş gen gösterdi. Cytoscape sonuçlarına göre, en iyi 15 hubba düğümü PTPRC, ITGAM, CCL2, ICAM1, MMP9, CXCL8, TLR2, CD86, CXCR4, IL1A, CD44, CCL3, ITGAX, CXCL10 ve CCR7 dahil olmak üzere sıralandı. Fonksiyonel olarak gruplandırılmış ağlar, bu genlerin esas olarak kemokin aracılı sinyal yolunda, endotel morfogenezinde ve nöroinflamatuar yanıtta zenginleştiğini belirledi. Sonuç: Araştırmamızda pulpitiste tanısal biyobelirteç olarak 15 gen tanıtılmış ve bunların fonksiyonel olarak gruplandırılmış ağları oluşturulmuştur. Ancak elde edilen sonuçların in vitro ve in vivo yöntemler kullanılarak doğrulanması gerekir. Anahtar Kelimeler: diş pulpası; iltihaplanma; Gen ontolojisi; DEG'ler; ÜFE; Pulpitis.*

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Peer-review: Externally peer-reviewed.

Author contributions: AA participated in designing the study. AA participated in generating the data for the study. AA, FSM, AM participated in gathering the data for the study. AA participated in the analysis of the data. SBB AA, FSM, AM wrote the majority of the original draft of the paper. AA, FSM, AM participated in writing the paper. AA, FSM, AM has had access to all of the raw data of the study. AA, FSM, AM has reviewed the pertinent raw data on which the results and conclusions of this study are based. AA, FSM, AM have approved the final version of this paper. AA guarantees that all individuals who meet the Journal's authorship criteria are included as authors of this paper.

Conflict of Interest: The authors declared that they have no conflict of interest.

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