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Original Article

Biochemical Pathway and Protein-Network Analysis of Dental Caries Based on Systems Biology Approaches

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Abstract

Background: Tooth decay (TD) is a multifactorial disorder, and several factors are involved in its etiology.
Objective: The present study aimed to unravel the main genes and molecular mechanisms underlying TD.
Methods: The dataset GSE1629 in the Gene Expression Omnibus (GEO) database was analyzed to uncover differentially expressed genes (DEGs) in patients with TD compared to patients with sound teeth. A protein-protein interaction network was built, and the most important clusters, hub genes, transcription factors (TFs), and protein kinases involved in the regulation of TFs were disclosed. Signaling pathways and Gene Ontology terms dysregulated in TD were also identified.
Results: A total of 196 DEGs were determined (false discovery rate < 0.001; |Log2 fold change| > 1). *PTPRC*, *ITGB2*, *TYROBP*, *MMP9*, *CXCL8*, *CD44*, *CCL2*, *C1QB*, *C3*, and *SPP1* were considered hub genes. Further, *BPTF* and *MAPK1* were demonstrated to be the highest TFs and protein kinases likely involved in the pathogenesis of TD, respectively.
Conclusion: *PTPRC*, *ITGB2*, *TYROBP*, *MMP9*, *CXCL8*, *CD44*, *CCL2*, *C1QB*, *C3*, *SPP1*, *BPTF*, and *MAPK1* may be regarded as potential markers for the therapeutic purposes of TD.
Keywords: Biomarker, Dental caries, Gene regulatory network, Protein-protein interaction network, Tooth decay

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Background

According to a report about tooth decay (TD), the Global Burden of Disease study estimated that more than two billion of the world's population are suffering from this highly prevalent chronic disorder (1). TD has remained a dominant global health issue in most modern and developing countries, in which 60%–90% of schoolchildren and a considerable number of adults have already been affected by this disease (2,3). TD is a comprehensive disorder, and various factors are involved in its occurrence. The pathogenesis of the disease can be elucidated by a logic diagram, which includes three circles and the interactions between them. The circles represent microbial load (plaque), intake of food, as well as host factors (e.g., genetic factors). The interaction among these circles could lead to dental caries (DCs). The parameter of "time" has also been added to the above circles, which displays the duration of interaction between caries-associated factors. Previous studies have shown that *Streptococcus mutans* and lactobacilli are primarily involved in the beginning and development of DC, respectively. Fermentable sugars are the primary substrates for these bacteria, and the carbohydrates produced from bacteria are mostly stable in the biofilm. DC could cause other oral disorders such as tooth pain, tooth defects, loss of teeth, and tooth crowding (4-12). However, the dentine–pulp complex has been reported to have regenerative characteristics by secreting a tertiary dentine extracellular matrix following a tooth injury, protecting the cells beneath the lesion, and maintaining the vitality of the tissues (13). Since previous studies have documented that the inflammatory reaction occurs due to bacterial infection in DC (14-21), further investigation is inevitable to illustrate the exact molecular mediation.

Reanalyzing the microarray datasets containing enormous gene expression data provides an opportunity to discover new genes previously not linked with the disorder and, therefore, could identify new potential biomarkers for diagnosis and curative aims. In addition, identifying novel caries-associated genes could help understand the etiology of this disorder (22,23). It may be hypothesized that the significant alteration in the expression of various genes in patients with DC compared to the individuals with sound teeth results in the deregulation of several

signaling pathways and biological processes (BPs) associated with the pathogenesis of DC. Moreover, the most critical genes and their regulating markers involved in the etiology of DC could be identified by analyzing the protein-protein interaction (PPI) network and gene regulatory network (GRN) associated with the disease, respectively (24). Kinase enrichment analysis also provides an opportunity to illustrate protein kinases involved in the phosphorylation of transcription factors (TFs) regulating the critical genes (25).

The present study, therefore, aimed to determine (1) differentially expressed genes (DEGs) within the pulpal tissue obtained from healthy and severely carious human teeth, (2) the hub genes in the PPI network, (3) the most significant TFs regulating the hub genes, (4) protein kinases involved in the TF's activity, and (5) signaling pathways and gene ontology (GO) in patients with tooth caries compared to individuals with sound teeth. This was performed by reanalyzing the gene expression dataset GSE1629 (22).

The gene dataset GSE1629, containing almost 15 000 human sequences, was established by McLachlan et al (22) to identify genes associated with carious lesions to promote diagnostic and therapeutic approaches. McLachlan et al (22) performed their study by utilizing pooled RNA extracted from patients with DC and individuals with sound teeth extracted from the pulpal region. The completely decayed and healthy premolar and molar teeth, collected for orthodontic aims from individuals from age 20 to 30 years, were extracted from the Birmingham Dental Hospital. DC illustrated the carious lesions varying from enamel to decays. Next, the extracted teeth were instantly overwhelmed in RNA stabilizing buffer using the RNA Later (Sigma, UK), and the complete tissue of the pulp was cautiously eliminated by using a sterile dental probe and forceps (26).

Materials and Methods

Microarray Expression Dataset Analysis

The dataset GSE1629 (2) was chosen for analyses from the Gene Expression Omnibus (GEO) (NCBI GEO, http://www.ncbi.nlm.nih.gov/geo) (27). The GSE1629 consisted of four pooled pulpal tissue samples from 11 patients with clinically DC showing deep dentinal lesions/pulp exposure (n = 2) and 12 with sound teeth (n = 2). This dataset was developed on the platform of Affymetrix Human Genome U133A Array (GPL96). DEGs between pulpal tissue of patients with DC compared to individuals with sound teeth were identified using the GEO2R tool (28) with the benchmark of $|\text{Log2 fold change(FC)}| > 1$ as well as the false discovery rate (FDR) < 0.001. Then, the Benjamini and Hochberg procedure was applied to calculate corrected *P* values.

The Protein-Protein Interaction Network and Clustering Analyses

The Search Tool for the Retrieval Of Interacting Genes (STRING) knowledge database (version 11.0 STRING, http://string-db.org) (29) was utilized for identifying possible connections between DEGs. Next, unconnected genes were removed from the network. The network analyzer tool within the Cytoscape 3.9.0 (http://www.cytoscape.org/) (30) was used for calculating the centralities (e.g., degree and betweenness) of nodes, while clustering analysis was carried out using the molecular complex detection (MCODE) plugin. The benchmarks considered for each module to be statistically significant included the number of nodes > 10, Degree cutoff = 2, MCODE score > 3, Max depth = 100, and the k-score = 2. MCODE is frequently used for determining condensed zones in PPI networks named clusters (modules) (31). It has been demonstrated that clusters include genes that take part in common pathways or BPs. Moreover, most of the clusters contain a seed node known to be a vertex of the modules based on its existing biological role in living systems (32-34).

Functional Analysis

The GO analysis is frequently used for mining particular biological properties from a set of high-throughput data, including genome, transcriptome, and proteome (35). The Reactome database (https://reactome.org/) provides perceptive bioinformatics tools for illustrating and analyzing the pathways to support theoretical and experimental study and genomics data mining, which has been developed at the Ontario Institute for Cancer Research, Cold Spring Harbor Laboratory, the New York University of Medicine, and The European Bioinformatics Institute (36). In addition, Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) is an online bioinformatics database providing an entire set of functional annotation tools for researchers to realize the biological concepts of multiple proteins and genes (37,38). DAVID was used to illustrate the DEGs enrichment of cellular components (CCs) and molecular functions (MFs) as well as the main clusters enrichment of BPs. In addition, the Reactome database was used to demonstrate pathways significantly associated with the salient modules in the PPI network. The cutoff conditions for GO annotations and pathways were set to FDR < 0.05 and the enriched gene numbers ≥ 2.

Master Regulators of the Hub Genes

The iRegulon plugin was utilized for the prediction of upstream TFs linked to the hubs. Only TFs with a normalized enrichment score (NES) > 3.0 were considered to be statistically significant (39). The NES shows the significance of the detected TFs and a correlation with the area under the curve value associated with the motifs (40-42). Subsequently, significant motifs (e.g., GRN) consisting of TFs and their downstream hub genes were built using the iRegulon plugin. A GRN provides valuable information associated with the upstream regulators of critical genes involved in the pathogenesis of the disease.

Therefore, GRN analysis might lead to identifying several crucial genes regulated with common TFs. Thus, future studies could recognize TFs as novel drug targets (43).

Consensus Sequences of Transcription Factors

JASPAR is an open-access database (https://jaspar.genereg.net/) (44) providing helpful information about TFs binding sites across six taxonomic classes, including fungi, insect, nematode, Plantae, Urochordata, and Vertebrata. This useful web server includes consensus sequences Logos and position frequency matrixes of several TFs. Position frequency matrixes illustrate the occurrences of nucleotides at each position in a set of demonstrated TF-DNA interactions and could be used for constructing position-specific scoring matrixes. The consensus sequences of these matrixes are made of nucleotides with the highest frequency at each position. The consensus sequences matching scores for top-ranked TFs were calculated in R programming (version 4.0.0) (45) based on the method described by Xiong (46). The probability of consensus sequences matching the binding sites of TFs is described as $2^{(\text{match score})}$ times more than that by random chance (46).

Protein Kinases Enrichment Analysis

Protein kinases add a phosphate group from adenosine triphosphate to three residues of proteins, including Thr, Ser, and Tyr, while phosphatases catalyze the reverse reaction. Phosphorylation and dephosphorylation of proteins affect the activity, localization, stability, and interaction of substrates with other molecules in biological networks. The abnormal expression or activity of these two classes of enzymes could affect several signaling pathways and BPs in human cells, resulting in many disorders (47-49). Here, the online kinase enrichment analysis 3 (KEA3) knowledge database (https://maayanlab.cloud/kea3/) (50) was utilized for uncovering upstream protein kinases potentially involved in the phosphorylation of TFs involved in the hub gene regulation. The KEA3 uses several types of resources to illustrate upstream TFs for a set of genes (proteins). These resources are based on PPI, kinase-substrate interaction, co-occurrence, and transcript co-expression (50). In this regard, the PPI libraries are as follows: BioGRID (51), mentha (52), huMAP (53), prePPI (54), MINT (55), HIPPIE (56), PIPs (57,58), PSOPIA (59), REACTOME (60), ChengPPI (61), and STRING (62). In addition, PhosphoSitePlus (63), PhosD (64), PhosphoNetworks (65), PTMsigDB (66), ChengPPI (61), and Phospho.ELM (67) are libraries used to study kinase-substrate interactions. After a gene set is uploaded into the KEA3, upstream protein kinases are scored and ranked based on different algorithms used in different libraries. KEA3 then calculates the mean and the sum of the ranks in different libraries.

Results

The Identification of Differentially Expressed Genes in Patients With Dental Caries

In our present study, we obtained the data of four pooled samples from the pulp tissue of 11 patients with DC showing deep dentinal lesions/pulp exposure (n = 2) and 12 individuals with sound teeth (n = 2). A total of 196 DEGs, including 146 overexpressed and 50 underexpressed genes, with an FDR less than 0.001 and the $|\text{Log2 FC}| > 1$ were identified using the GEO2R online tool and considered for further analysis in the present study. Top-ranked over- and underexpressed genes in carious teeth compared to those in the sound teeth are presented in Figure 1, and Figure 2a demonstrates the volcano plot of DEGs. Figure 2b illustrates the hierarchical clustering of the hub genes, and Figure 2c demonstrates the interactions between hub genes obtained from the DAVID database.

Protein-Protein Interaction Network, Clustering, and Functional Analyses

A PPI network was achieved based on the STRING database's DEGs, and the cut-off for confidence score was set to ≥ 0.4. After removing disconnected nodes, a PPI network with 141 genes and 917 connections was transferred into the Cytoscape software for advanced analyses.

The MCODE plugin identified two substantial clusters (i.e., cluster No. 1 and cluster No. 2) within the PPI network (Figure 3). These clusters were involved in the pathways and BPs associated with DC. They consisted of several genes mainly enriched in pathways and BPs linked to the human immune system, including 'antigen processing and presentation of peptide or polysaccharide antigen via major histocompatibility complex (MHC) class II (BP)', 'immune response (BP)', 'antigen processing and presentation of exogenous peptide antigen via MHC class II (BP)', 'antigen processing and presentation (BP)', 'inflammatory response (BP)', 'leukocyte migration (BP)', 'interferon-gamma-mediated signaling pathway (BP)', 'T cell costimulation (BP)', 'chemotaxis (BP)', 'positive regulation of T cell activation (BP)', 'MHC class II antigen presentation (Pathway), 'T cell receptor (TCR) signaling (Pathway), and 'interferon-gamma signaling (Pathway)'.

At an FDR less than 0.05, a total of 35 pathways and 14 BPs were found to be significantly affected in DC. Moreover, DAVID analysis revealed that a total of 26 CCs and 10 MFs were significantly dysregulated in patients with DC. Figure 4 illustrates the top 10 significant pathways, BPs, CCs, and MFs enriched in DC. Accordingly, the most meaningful BPs and pathways were linked to the human immune system, including 'antigen processing and presentation of peptide or polysaccharide antigen via MHC class II' and 'MHC class II antigen presentation (Pathway)'. Furthermore, the average centrality value within the PPI network for betweenness and degree

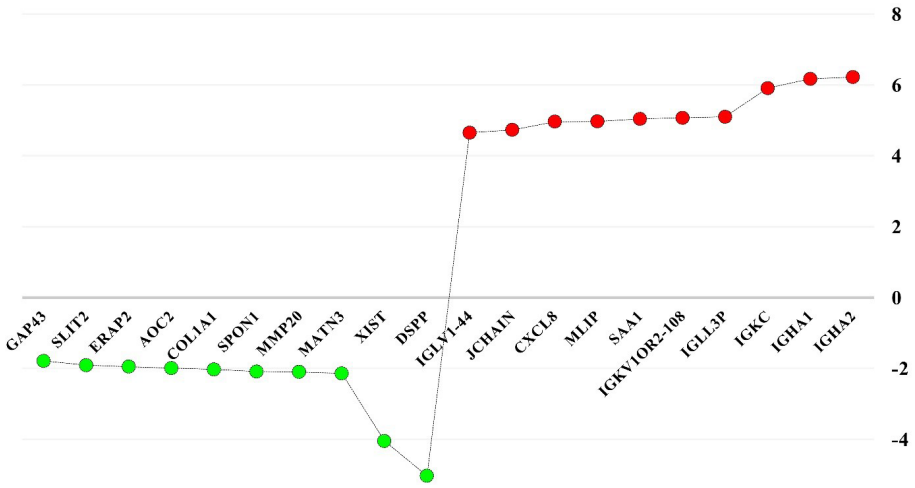

Figure 1. Top-10 Up- and Downregulated Genes in Pulpal Tissue of Patients With Carious Lesion Compared With the Healthy Pulp Tissue Regarding Their Log2 FC. *Note.* FC: Fold change. The x-axis shows the genes' name, while the y-axis demonstrates the value of Log2 FC

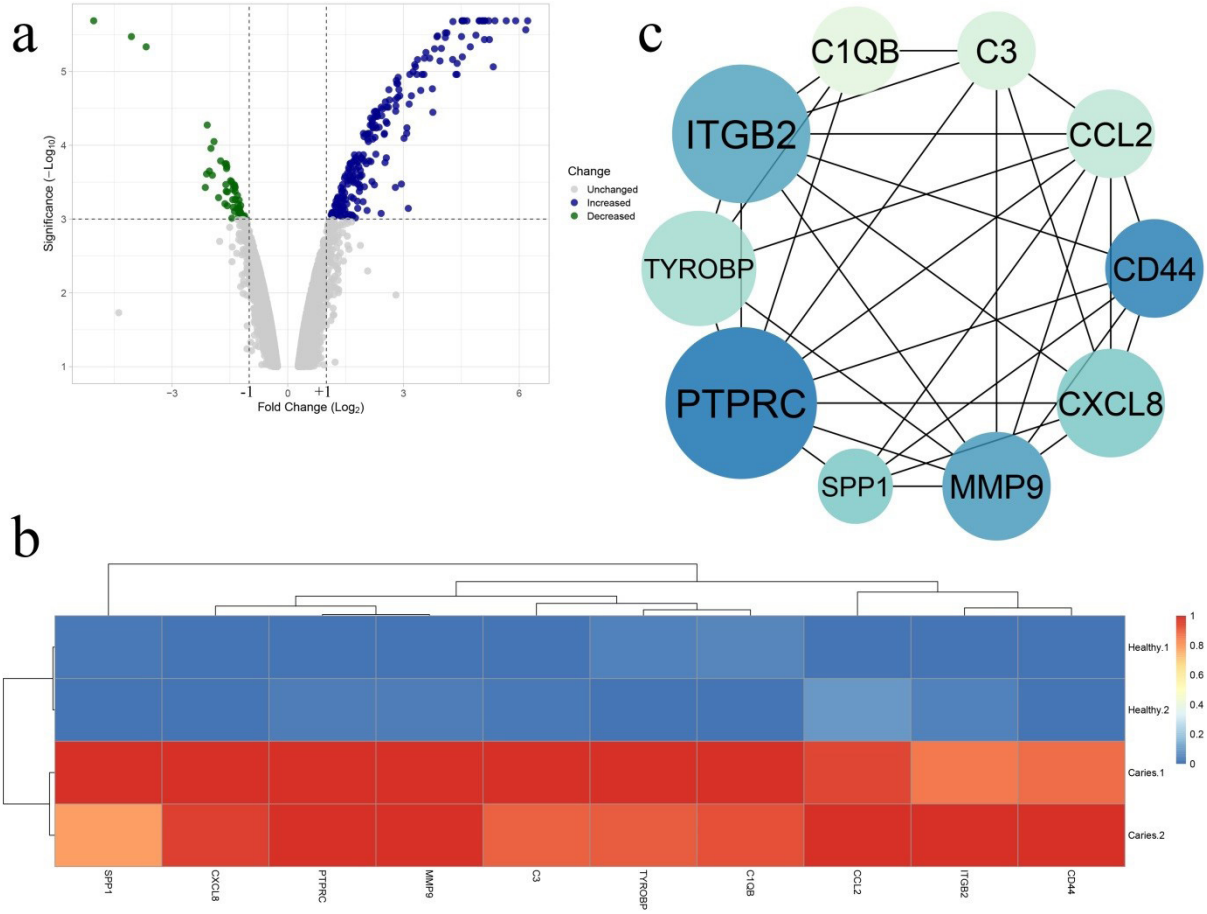

Figure 2. (a) Volcano Plot of Genes in the Dataset GSE1629. (b) Hierarchical Clustering of the Hub Genes. (c) Interactions between the Hub Genes. *Note.* PPI: Protein-protein interaction. More connected genes in the main PPI network are presented larger, while the color of the nodes is linked to the betweenness centrality value

was calculated as 0.012620178 and 13.0071, respectively. Accordingly, a total of 10 genes were revealed to have a degree and betweenness values more than twice of the nodes within the network, and therefore, were considered hub genes associated with the pathogenesis of TD (Table 1).

Master Regulators of Hub Genes

The iRegulon plugin was executed to predict TFs regulating the hub genes. Only TFs with the criteria of NES > 3.0 were considered to be statistically meaningful (39). Accordingly, there were 50 TFs significantly associated with tooth caries. The most enriched TF was bromodomain and PHD finger-containing transcription factor (*BPTF*) with an NES equal to 7.167. A total of four hubs were found to be downstream genes of *BPTF* including *ITGB2*, *C1QB*, *CD44*, *PTPRC*, and *C1QB*. The

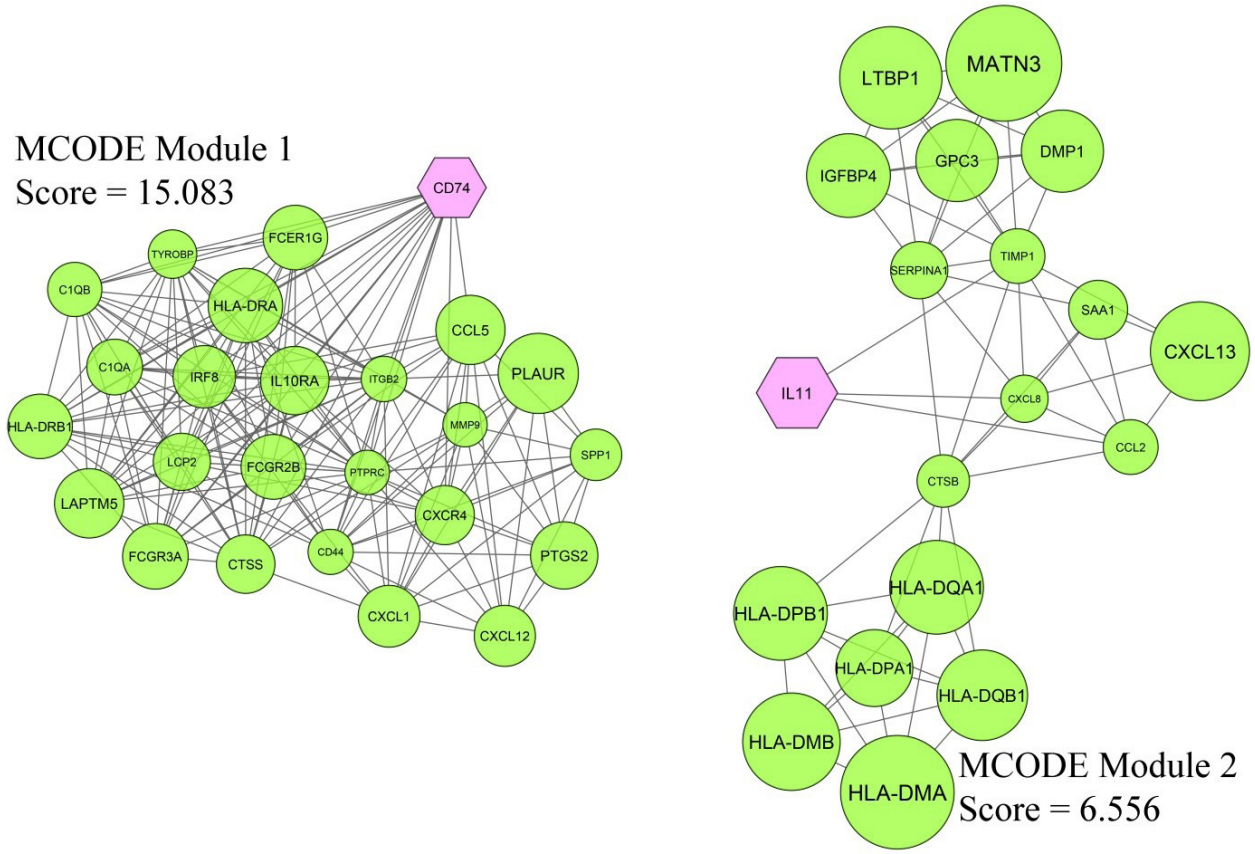

Figure 3. Module Analysis. *Note.* PPI: Protein-protein interaction; DEG: Differentially expressed genes; MCODE: Molecular complex detection. The network was built based on the DEGs in patients with dental caries compared to patients with sound teeth. The MCODE plugin discovered two salient clusters in the PPI network. The Hexagons illustrate seed nodes in each module. The size of the nodes is directly correlated with the clustering coefficient value of the genes in the leading PPI network

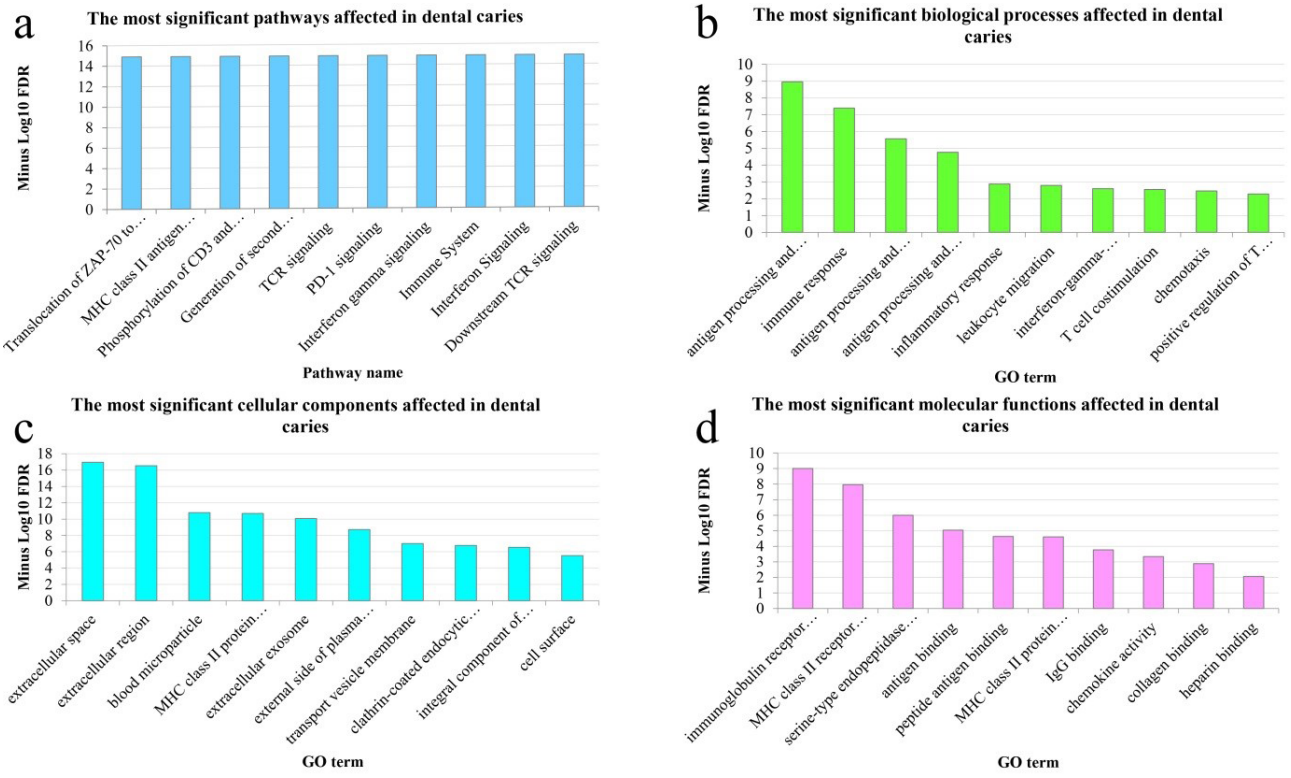

Figure 4. Top-10 (a) Pathways, (b) Biological Processes, (c) Cellular components, and (d) Molecular Functions Enriched in Patients with DCs Concerning Their FDR. *Note.* DCs: Dental caries; FDR: False discovery rate. The genes' names are presented at the top left corner of the Logos. The consensus sequences scores are also demonstrated below the names. The x-axis shows the name of the term, and the y-axis corresponds to the Log10 FDR

Table 1. A Total of 10 Hub Genes With the Criteria of Degree and Betweenness More Than Twice the Average of the Nodes in the PPI Network Associated With DCs

Gene ID	Degree	Betweenness
PTPRC	53	0.0924642
ITGB2	49	0.07415381
TYROBP	42	0.04844631
MMP-9	40	0.07775514
CXCL8	40	0.06116186
CD44	37	0.08761097
CCL2	34	0.03807377
C1QB	34	0.02585173
C3	31	0.03080516
SPP1	30	0.06125618

Note. ID: Identification; DCs: Dental caries; PPI: Protein-protein interaction; ITGB2: Integrin beta 2; MMP: Matrix metalloproteinases.

statistics of the significant TFs are presented in Table 2.

Binding Sites Logos and Matching Scores of Transcription Factors

The top-ranked TFs with the criteria of NES > 5 were investigated in the JASPAR webserver. The consensus sequences Logos of nine TFs, including *TCF12, SPI1, ESR2, STST3, RELB, PAX5, RXRA, TCF15, and HNF4A*, were available in the database. The maximum and minimum scores for TFs binding sites were calculated at 22.91 and 7.5 for *ESR2* and *SPI1*, respectively (Figure 5).

Upstream Protein Kinases

Top-10 protein kinases based on the mean of the ranks in different libraries were considered significant. Moreover, all top-10 ranked kinases had a *P* value less than 0.05. Mitogen-activated protein kinase 1 (*MAPK1*) demonstrated the best result with the mean rank score and a *P* value of 19.64 and 6.96E-05, respectively (Figure 6 and Table 3). Finally, a GRN was constructed based on the eight hub genes, 50 TFs, and 10 protein kinases. This regulatory network included 440 edges (Figure 7).

Discussion

DC is a common chronic disease affecting more than 60% of childhood and a considerable percentage of adults within the most industrialized and developing countries. It is a multifactorial disorder, and several agents are involved in its pathogenesis, including cariogenic bacteria and genetic factors (4-9). Despite many types of study in this scope, our knowledge regarding the molecular mechanisms and most essential genes that participate in the beginning and development of TD is limited (22).

In the present study, we tried to determine potential biomarkers, master regulators, protein kinases regulating TFs, signaling pathways, and BPs associated with the pathogenesis of DC. Therefore, gene expression data using pooled RNA from the pulp tissue of 12 individuals with

Table 2. The 50 TFs Significantly Identified for Regulating Hub Genes in DCs

TF	Targets	NES
BPTF	ITGB2, CD44, PTPRC, C1QB	7.167
TROVE2	C1QB, C3, CD44, SPP1, PTPRC, CCL2	7.113
TCF12	C1QB, C3, ITGB2, CD44	6.472
SPI1	C1QB, PTPRC, C3, CD44, ITGB2, TYROBP	6.027
CBFB	SPP1, ITGB2, TYROBP	5.878
ESR2	ITGB2, C3, C1QB, SPP1, CD44	5.419
STAT3	PTPRC, C3	5.402
RELB	C3, CD44, ITGB2, C1QB, CCL2, PTPRC	5.355
PAX5	PTPRC, ITGB2	5.319
RXRA	CD44, ITGB2, C3, CCL2, TYROBP, C1QB	5.155
TCF15	SPP1, CD44, ITGB2, PTPRC, C1QB	5.133
ZNF691	CCL2, C3, CD44, ITGB2, PTPRC, C1QB, TYROBP	5.124
HNF4A	ITGB2, C3	5.008
PREB1	C1QB, ITGB2	4.97
TCF7L2	CCL2, C1QB	4.881
FOXO3	PTPRC, ITGB2, CCL2, SPP1, CD44	4.861
EP300	ITGB2, C1QB, PTPRC, CD44, CCL2	4.506
TFAP2C	SPP1, C3, ITGB2	4.478
SPIB	CD44, CCL2, C3, C1QB, TYROBP, ITGB2, PTPRC	4.461
JAZF1	C1QB, ITGB2, CD44	4.429
TFAPA2	ITGB2, SPP1, C3	4.421
HSF1	CD44, PTPRC, CCL2, C1QB, C3, ITGB2	4.402
PAX8	TYROBP, ITGB2, C3	4.347
GATA1	C1QB, ITGB2, PTPRC	4.343
PROX2	ITGB2, CCL2, CD44, C1QB, SPP1, TYROBP	4.338
MEF2A	ITGB2, CD44	4.311
TBL1XR1	ITGB2, CD44	4.307
TEAD4	ITGB2, CCL2	4.198
GFI1B	C3, CCL2	4.184
POLE3	SPP1, C1QB, ITGB2, CD44, PTPRC	4.179
POLR3A	CD44, C1QB	4.099
TRMT1	C1QB, SPP1, ITGB2, C3, CD44	4.08
HNF4G	ITGB2, CCL2, C3, C1QB	4.061
DBP	CCL2, SPP1	4.011
SP1	C3, ITGB2	4.004
NFKB1	CD44, ITGB2	3.966
HOXB8	C3, ITGB2, PTPRC, SPP1, C1QB	3.821
STAT1	CCL2, C3	3.801
CELF5	C1QB, ITGB2	3.684
NFIC	CD44, CCL2, ITGB2	3.677
TFAP2B	PTPRC, ITGB2, SPP1, C1QB	3.644
HNRNPH3	C3, CCL2, C1QB	3.621
SMAD5	ITGB2, SPP1	3.566
GTF3C2	ITGB2, C1QB, C3	3.462
AVEN	PTPRC, CD44, SPP1, CCL2, ITGB2	3.457
ARID3A	ITGB2, C1QB, PTPRC	3.348
TGIF2LY	CCL2, PTPRC, ITGB2, C3, C1QB	3.221
GCM1	ITGB2, C1QB, C3, CCL2, SPP1	3.14
SMC3	TYROBP, CD44	3.133
RAB7A	C3, C1QB, ITGB2, TYROBP	3.09

Note. DCs: Dental carries; *BPTF:* PHD finger-containing transcription factor; TF: Transcription factor; NES: Normalized enrichment score.

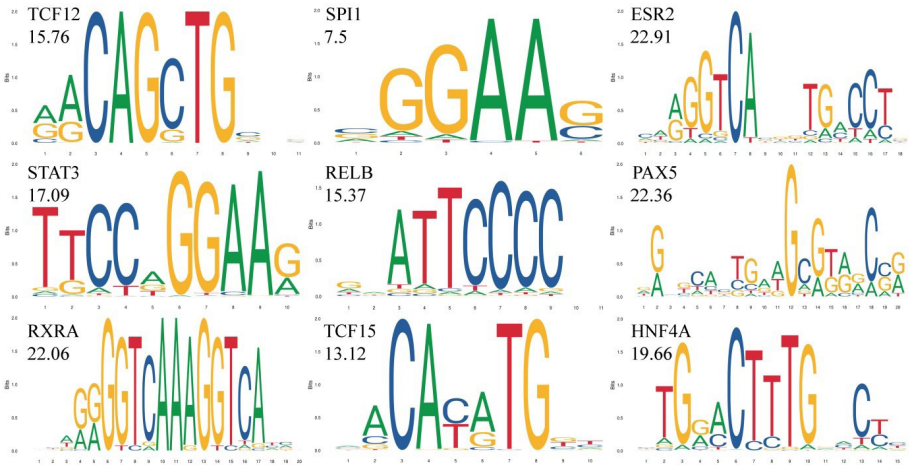

Figure 5. Consensus Sequences Logos of Top-ranked TFs Regulating the Hub Genes in DCs. *Note.*TF: Transcription factor; DCs: Dental caries; FDR: False discovery rate. The genes' names are presented at the top left corner of the Logos. The consensus sequences scores are also demonstrated below the names

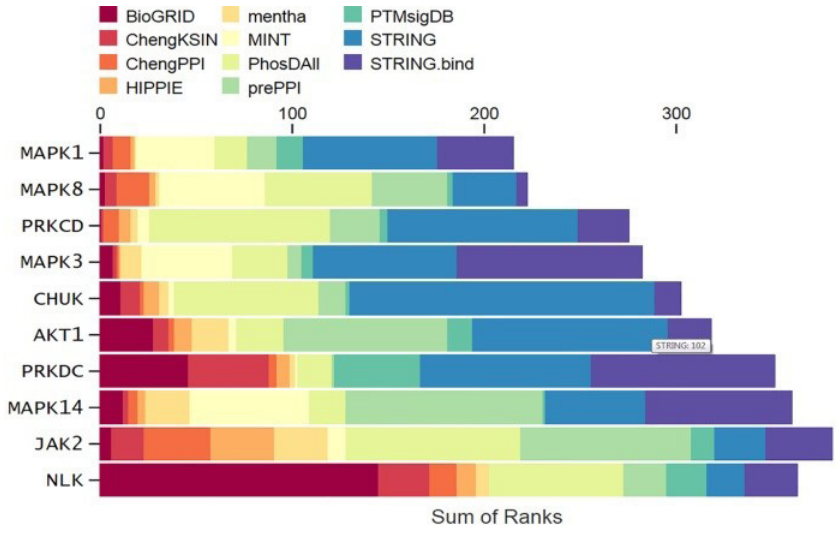

Figure 6. The Mean Rank Visualization from KEA3 for TFs Regulating the Hub Genes. *Note.* KEA3: Kinase enrichment analysis 3; TF: Transcription factor. The x-axis demonstrates the sum of ranks in different libraries, while the y-axis shows the protein kinases' names

Table 3. Top-ranked Protein Kinases Involved in the Phosphorylation of TFs Regulating the Hub Genes

Protein kinase	Mean Rank	*P* Value
MAPK1	19.64	6.96E-05
MAPK8	20.27	1.35E-04
PRKCD	25.09	1.35E-06
MAPK3	25.73	1.50E-06
CHUK	27.55	6.89E-04
ATK1	29	2.35E-04
PRKDC	32	1.35E-06
MAPK14	32.82	3.55E-06
JAK2	34.73	3.69E-03
NLK	36.4	2.10E-02

Note. TFs: Transcription factors; *MAPK1:* Mitogen-activated protein kinase 1.

clinically sound teeth and 11 patients with DC exhibiting deep dentinal lesions/pulp exposure were obtained from the GEO web tool and analyzed. A total of 196 DEGs, including 146 overexpressed and 50 underexpressed genes, with the criteria of FDR < 0.001 and the value of |Log2 FC| > 1 were identified. Further, the most significant pathways and BPs associated with DC were identified using the Reactome and DAVID online databases, respectively. Moreover, a PPI network associated with DC was built and analyzed based on DEGs.

The MHC includes a set of genes within the jawed vertebrates taking part in the immune system (68). The MHC is positioned at chromosome-6 (6p21.3) in humans and contains more than 200 genes and many alleles (69,70) and encodes glycoproteins named human leukocyte antigens (*HLAs*) that take part in the body's immune defense by the presentation of short peptides to T cells (71).

Various *HLA* alleles are involved in different human disorders and conditions such as autoimmune disease, inflammation, cancer, social behavior, as well as a shorter lifetime. However, the exact underlying mechanism has not been clearly understood. Overall, *HLAs* are classified into two main subclasses (i.e., *HLA* class I and *HLA* class

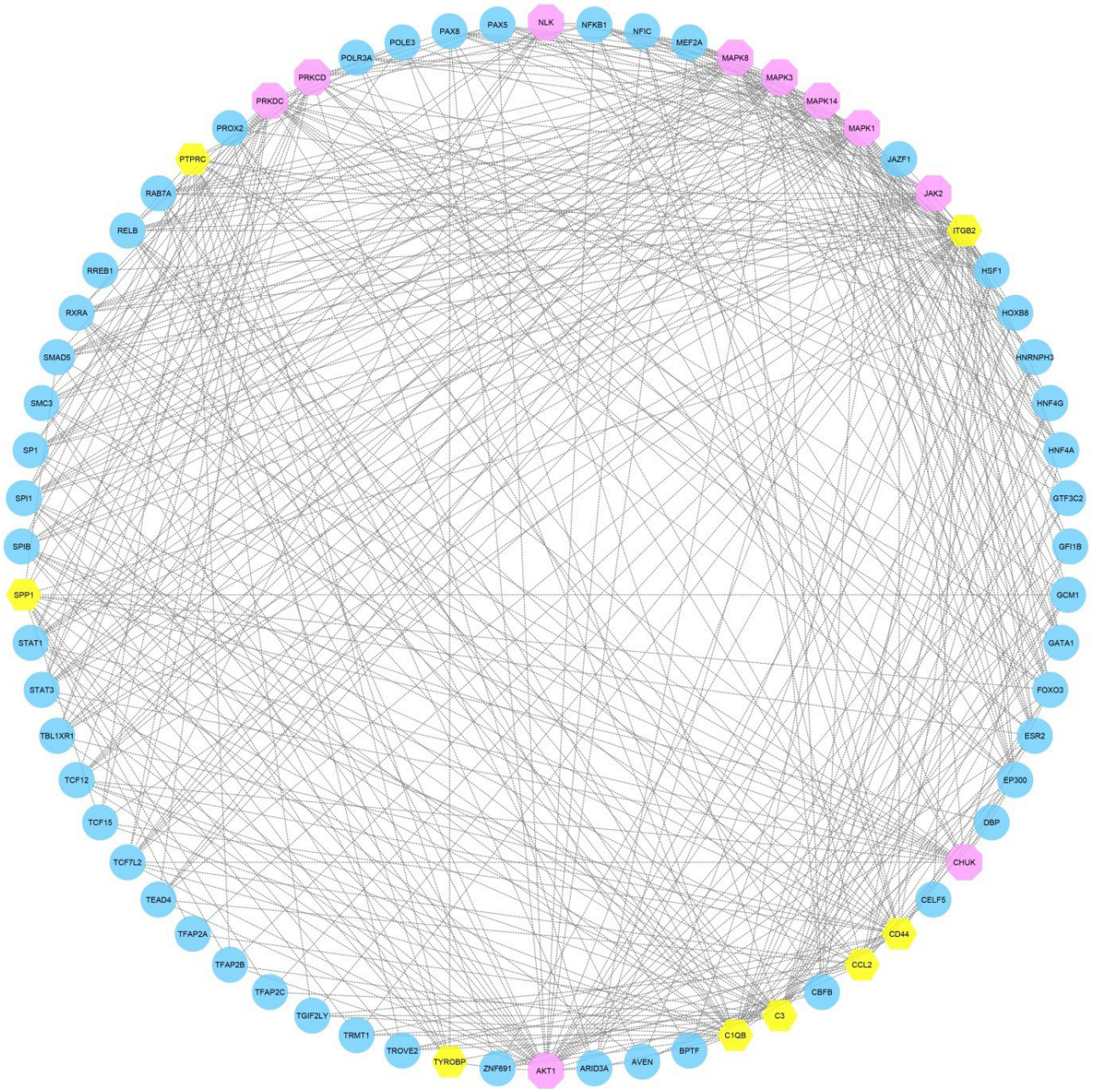

Figure 7. Gene Regulatory Network Associated with the Pathogenesis of DCs. *Note.* DCs: Dental caries. The yellow (hexagon), blue (circle), and violet (octagon) nodes represent hub genes, transcription factors, and protein kinases, respectively

II) based on their different structures, biological roles, and the expression on the surface of cells.

All nucleated cells contain different amounts of *HLA* class I proteins on their cell membrane. These proteins contribute to presenting peptides from degraded proteins or abnormal ones from hazardous viruses inside the cytoplasm to the TCR of the $CD8^+$T cells, contributing to activating the defense system. Furthermore, the *HLA* class II proteins are exhibited on the surface of B cells and the other antigen-presenting cells, known as active immune cells. These proteins are involved in presenting extracellular antigens to $CD4^+$T cells. Additionally, T cells produce *HLA-II* main isoforms, including *HLA-DP*, *HLA-DR*, and *HLA-DQ*, almost 3-5 days after activation. Furthermore, interferon-γ and interleukin (IL)-4 are produced just after a few hours following the activation of TCR (72-80).

TD occurs after the deterioration of the tooth's organic and/or mineral texture. Matrix metalloproteinases (MMPs) take part in collagen loss, while bacteria contribute to damaging the inorganic compounds within the healthy tissues of the teeth (81). *S. mutans* is the most prevalent caries-associated bacteria, but *Lactobacillus acidophilus* and *Lactobacillus casei* have also been linked with dental plaque (82,83).

A significant correlation has been observed between caries resistance and a robust immune system. In this regard, caries-free individuals revealed higher serum antibody titers to streptococcal antigens than caries-susceptible individuals (84). Further, caries-free individuals were

found to produce T cells with a salient ability to increase at the time of exposure to *S. mutans* antigens (85). Moreover, caries-resistant subjects can potentially generate more active T-helper-cells upon exposure to a minor amount of *S. mutans* antigens compared with patients exhibiting TD (86). Therefore, *HLA*-related genes from cluster 2 might enhance the immune system against *S. mutans* antigens and vice-versa.

It has also been reported that different *HLA* alleles may affect bacterium-induced TD. In this case, a significant DR-positive cell aggregation was observed within the pulp tissue of the patients with superficial caries (87). Moreover, the DR-positive cell aggregation was expanded to odontoblast cells in patients with deeper caries lesions (86).

Acton et al (88) performed a study to examine the possible correlation between MHC alleles at the *HLA-DRB1*, *DQB1*, and *TNFa* microsatellite loci and the number of oral bacteria associated with DC, in addition to the decayed, missing, and filled teeth index in 186 African-American females. The patients had an average age and median decayed, missing, and filled surface indexes of 20.8 years and 9 (range 0–68), respectively. Acton et al (88), reported a significant correlation between enhanced levels of *S. mutans* and the elevated expression of *DRB1**4 and *DRB1**3 (*P*-value = 0.005). Furthermore, the enhanced level of *DRB1**8 was linked to the increased level of *S. mutans* (*P* = 0.04). A positive correlation was also observed between the levels of *DRB1**1 and *L. casei* (*P* = 0.04). Moreover, the *TNFa* allele 103 and *TNFa* 117 were negatively and positively associated with the levels of *L. acidophilus*, respectively. Moreover, Acton et al (88) demonstrated a significant correlation between the production of *HLA* class II alleles, *TNFa* genetic, and the colonization of DC-associated oral bacteria, including *S. mutans*, *L. casei*, and *L. acidophilus*.

Based on the obtained results, a total of 25 nodes were involved in cluster 1 such as *HLA-DRB1* and *HLA-DRA*. Further, cluster 2 consisted of 19 genes, including *HLA-DPB1*, *HLA-DQA1*, *HLA-DMB*, *HLA-DQB1*, *HLA-DPA1*, and *HLA-DMA*. Further analysis revealed that the HLA isotypes mentioned above were significantly over-expressed in the pulp tissue of patients with tooth caries compared to patients with sound teeth with the benchmark of FDR < 0.001 and the absolute value of Log2 FC > 1. Accordingly, it may be hypothesized that these genes contribute to the beginning and/or developing of TD. Otherwise, the enhanced expression of these genes may be due to the response of increased tooth caries. However, more experiments are necessary to approve our findings.

A total of 10 genes demonstrated degree and betweenness values higher than twice the average of the genes within the PPI network and, therefore, were found to be hub genes playing a significant role in the etiology of DC. The top-ranked genes based on the degrees were *PTPRC* (*CD45*), *ITGB2*, *TYROBP*, and *MMP9*, while *PTPRC*, *CD44*, *MMP9*, and *ITGB2* revealed the highest betweenness centrality in the PPI network, respectively.

CD45 is a tyrosine-protein phosphatase antigen encoded by the *PTPRC* gene. It positively regulates T cells through binding to the dipeptidyl-peptidase 4 (*DPP4*) (89-91). Lacerda-Pinheiro et al (92) designed a study to understand the principles of the differentiation of pulp cells promoted by the bioactive molecules. The authors implanted the agarose beads (alone or covered with the products of the amelogenin gene [A + 4 and A-4]) in the mucosa of the cheeks in mice. They reported that agarose increased the recruitment of *CD45*$^{+}$ cells, leading to the enhanced recruitment of leukocytes from the vascular compartment, resulting in the enhanced re-differentiation of leukocytes into osteo-chondrogenic lineages. In addition, the coated beads with A + 4 induced the production of osteo-chondrogenic markers, including *RP59*, *SOX9*, and bone sialoprotein. It is worth mentioning that *RP59* is an antigen produced in the bone marrow and young osteoblasts (93,94). Moreover, *SOX9* is a TF that participates in chondrocyte differentiation and skeletal development (95). Our results revealed that *CD45* was significantly overexpressed in the pulp tissue of patients with DC compared to patients with sound teeth (FDR = 3.46E-05; Log2 FC = 2.8), which may be due to the response of increased TD.

MMPs are a family of zinc-dependent proteolytic enzymes taking part in the degradation of extracellular matrix proteins such as collagens in their native and denatured forms. *MMPs* are activated by decreasing the pH to 4.5, followed by neutralization. Previous studies have demonstrated that the dentin matrix mainly contains type I collagen, and *MMPs* have a specific role in DC pathogenesis (96). Several types of *MMPs* are involved in dentin matrix elimination, including *MMP8* (collagenase-2), *MMP2* (72-kD gelatinase), *MMP9* (92-kD gelatinase), *MMP13* (collagenase-3), and *MMP20*, leading to TD (81,97); therefore, the systemic application of *MMP* inhibitors may be helpful in the prevention of TD (96).

Wang et al (98) studied the correlation between the saliva levels of *MMP2*/*MMP9* and different stages of DC in childhood. The levels of *MMP2* and *MMP9* were measured by the enzyme-linked immunosorbent assay. Wang et al (98) reported that the saliva levels of *MMP9* in severe and mild-caries groups were significantly higher compared with the caries-free group (*P* value < 0.05). However, no significant difference was observed between the severe caries group and the mild caries group (*P* value > 0.05). Wang et al (98) concluded that the salivary levels of *MMP9* might be correlated with TD in children. According to our results, *MMP9* was significantly overexpressed in the pulp tissue of patients with TD compared to patients with sound teeth (FDR = 5.78E-05; Log2 FC = 2.21), suggesting that *MMP9* is involved in the etiology of DC.

Integrin beta 2 (*ITGB2*) is a type of integrin chain and has been reported to be produced explicitly in leukocytes, promoting the connection between leukocytes and the

endothelium (99,100). Zhang et al (101) compared the expression level of *ITGB2* in cancer-associated fibroblasts (CAFs) and normal fibroblasts in patients with oral squamous cell carcinoma (OSCC) by using the reverse transcription polymerase chain reaction and western blot analyses, demonstrating that the *ITGB2* expression significantly increased in CAFs compared with the matched normal fibroblasts. The authors reported that the over-expression of *ITGB2* caused the hyper-activation of glycolysis through PI3K/AKT/mTOR pathways, leading to enhanced lactate excretion in CAFs and more proliferation of OSCC cells. The hyperactivity of glycolysis was found and confirmed through bioinformatics approaches and gas chromatography/mass spectrometry analysis, respectively. According to the present results, a significant increase was found in the expression of *ITGB2* in the pulp tissue of patients with DC compared with patients with sound teeth (*P* value = 1.6E-04; Log2 FC = 1.97). We hypothesize that some of the mechanisms that mediate DC may be similar to those in OSCC, leading to more production and excretion of lactate from the pulp tissue into the dentin region, resulting in a lower pH; as a consequence, causing enhanced dental decay, although this requires confirmation.

A total of 50 TFs were determined as master regulators of eight of the hub genes. Bromodomain and *BPTF* were the most significant enriched TFs with the criteria of NES = 7.167, regulating *ITGB2*, *CD44*, *PTPRC*, and *C1QB*. *BPTF* is the central member of the human nucleosome remodeling factor (102). The misregulation of *BPTF* has been demonstrated in many human cancers such as bladder cancer (103), hepatocellular carcinoma (104), glioma (105), and lung cancer (106). However, the etiological role of *BPTF* in DC needs more studies in the future.

Moreover, *MAPK1* demonstrated the best score among protein kinases regulating TFs (mean rank = 19.64). *MAPKs* are intracellular protein kinases necessary for inflammatory bone loss by activating *MMPs* and inflammatory cytokines (107). In this regard, *p38 MAPK* is an upstream activator of TNF-a, IL-1b and -6, and prostaglandin E2 (108), leading to the overexpression of prostaglandins, *MMPs*, and receptor activator of nuclear factor kappa beta (109,110), resulting in osteoclastogenesis and bone loss procedures (107). Similar mechanisms might be involved in the aberrant expression of *MAPK1* in DC, leading to tooth loss; however, confirmation is needed.

Our study had certain limitations. Only individuals from the Birmingham Dental Hospital were included in the GSE1629 dataset; therefore, the present results may not entirely justify the data obtained from the other nationalities. Further, only four pooled pulpal tissue samples from 11 patients with clinically DC having deep dentinal lesions/pulp exposure and 12 individuals with sound teeth were included in the GSE1629 dataset, and the sample size was small. Hence, a greater number of individuals with DC and sound teeth may promote the reliability of statistical approaches and probably illustrate more DEGs that are significantly linked to the pathogenesis of carious teeth. Moreover, the genes profiled in this study were developed from the Affymetrix Human Genome U133A Array GPL96 platform, which probably does not represent all the genomes. In addition, our results were achieved based on bioinformatics analyses; therefore, considerable *in vitro* experiments are necessary to approve our findings. In future studies, molecular experiments with large targeted cohorts are obligatory to support our data.

Conclusion

Overall, the present study successfully identified 196 genes differentially expressed in the pulpal tissue of patients with tooth caries compared to patients with sound teeth (adjusted *P* value < 0.001; |Log2 FC > 1|). Furthermore, a total of 10 hub genes including *PTPRC*, *ITGB2*, *TYROBP*, *MMP9*, *CXCL8*, *CD44*, *CCL2*, *C1QB*, *C3*, and *SPP1* were determined as proteins of potential importance to the pathogenesis of DC in the PPI network. Further, *BPTF* and *MAPK1* were found to be the most significant master regulator and protein kinase potentially involved in the etiology of DC, respectively. Moreover, substantial modules in the PPI network were mostly enriched in the pathways and BPs linked to the immune system. However, more studies and wet-lab experiments must be carried out in the future to confirm these findings and demonstrate their definite role in the etiology of DC.

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Authors' Contribution

Conceptualization: Amir Taherkhani, Zeinab Mohamadi, Zahra Khamverdi.
Data curation: Amir Taherkhani.
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Supervision: Amir Taherkhani.
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Visualization: Amir Taherkhani.
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Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

The data sets included in the study were 'The Gene Expression Omnibus (GEO) database (NCBI GEO, http://www.ncbi.nlm.nih.gov/geo)', 'The Search Tool for the Retrieval of Interacting Genes (STRING) online database (version 11.0 STRING, http:// string-db.org)', 'Database for Annotation, Visualization, and Integrated

Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/)', and 'Reactome online database (https://reactome.org/)'.

Ethical Approval

The present study has been confirmed by the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (Ethics no. IR.UMSHA.REC.1399.493).

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