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ORIGINAL RESEARCH ARTICLE

Identification of Genetic Alterations in Periodontitis Patients with Poorly Controlled Type 2 Diabetes Mellitus

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Abstract

Purpose: Periodontitis and diabetes are highly prevalent chronic diseases associated with upregulated inflammation that may adversely affect each other. The aim of this study was to determine underlying molecular mechanisms via bioinformatic tools as a guide for future studies.
Materials and Methods: Expression data (GSE156993) of Type 2 Diabetes Mellitus (T2DM) and Periodontitis (P) patients was selected from the Gene Expression Omnibus (GEO) database. Study groups were defined as follows; T2DM-poor (HbA1≥8.5%, n=7), T2DM-well (HbA1c<7.0%, n=7) and P (n=6). The differentially expressed genes (DEGs) between groups were analyzed with GEO2R software (log2FC≥0 or ≤0). Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for the identification of biological pathways. Protein network was constructed in STRING database and hub genes were detected. Data validation was performed via ELISA assay for two hub genes. Significance was set to P<0.05.
Results: 1008 genes were upregulated, while 610 genes were downregulated in T2DM-poor group compared to the controls. KEGG analysis revealed that the highest number of downregulated genes were clustered in cancer pathways and PI3K/Akt signaling pathway, as upregulated genes were clustered in purine metabolism, parathyroid hormone metabolism, cGMP/PKG signaling pathway and Rap1 signaling pathway. For increasing and decreasing expression profiles, hub nodes with the highest score were selected as SMAD4, HNF4A, SMARCA4 and SRC, TNF, RFC2, RFC3 genes, respectively.
Conclusions: Bioinformatic analyses revealed that metabolomic, inflammatory and cancer pathways were altered in periodontitis patients with poorly controlled diabetes. As protein-protein interactions may differ in vivo, further validation of the presented data is needed.

Key words: Type 2 Diabetes Mellitus; Periodontitis; Bioinformatic Analysis; GEO; Microarray

Introduction

Periodontitis is a chronic inflammatory disease that involves breakdown of the tooth supporting tissues such as gingiva, periodontal ligament and alveolar bone.[1,2] It is associated with accumulation of dental plaque biofilm, followed by microbial dysbiosis which is characterized by a long-lasting and destructive inflammatory response. Diabetes mellitus is a systemic disease associated with hyperglycemia, which may damage the eyes and the nervous, cardiovascular, and renal systems, and impair wound healing.[3] Several studies suggest that diabetes is also a risk factor for periodontitis or course of the disease is more severe in diabetic patients.[4–6] According to Meales et al., when age, sex etc. factors are excluded, risk of periodontitis is three-fold higher in diabetic patients than non-diabetic ones.[7]

Although mechanisms that link the two diseases are imprecise, each condition is associated with upregulated inflammation that may negatively affect each other.[2] In diabetic patients, it has been demonstrated that advanced glycation end-products (AGEs) are deposited in periodontal tissues, leading to activation of local immune and inflammatory reactions.[8] These upregulated reactions lead to bone resorption due to increased secretion of cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6.[9,10] Inflamed periodontal tissues then contribute to systemic inflammation, together with periodontal bacteria and their products. Chronic systemic inflammation results in impaired insulin signaling and insulin resistance, where HbA1c levels increase, leading to an additional negative impact on the periodontal inflammation. As bi-directional relationship between two diseases is based on complicated feedback mechanisms, understanding the molecular mechanisms is crucial for the proper management of periodontitis in diabetic patients.

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Several cytokines were nominated as controlling factors of the pathogenesis of both diseases, but the common genetic pathways may not be directly related to inflammation and are yet to be discovered.[2] Bioinformatic analyses may reveal the candidates, as transcriptomic data from patients suffering from both diseases are available. Many studies were conducted with datasets from one of two diseases,[11–13] as well as bioinformatic studies with data from both diseases.[14,15] Our study is distinct from the literature, as rather than shared genes and common pathways, we have focused on alterations in chronic periodontitis when diabetes is poorly controlled.

Material and Methods

Microarray data

Expression data with accession number GSE156993 of Type 2 Diabetes Mellitus (T2DM) and Periodontitis (P) patients was selected from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) database. Inclusion criteria was i) patients should have both diseases at the same time ii) microarray gene expression data must be present iii) gene expression analyses must be present. The GSE156993 data set was acquired by transcriptomic analysis of patients' peripheral blood mononuclear cells (PBMC). Patients with increased HbA1c levels ($\geq$8.5%) were defined as poorly controlled (T2DM-poor, n=7), while patients with low HbA1c (<7.0%) levels were defined as well-controlled T2DM groups (T2DM-well, n=7). Patients with periodontitis, but not T2DM, were used as a control (P, n=6). Affymetrix Human Genome U133 Plus 2.0 Array was used in the study.

Data processing

The differentially expressed genes (DEGs) between groups in the dataset were analyzed with GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r), an online tool that compares two sets of samples in the GEO dataset. DEGs with a P value of <0.05 and log2FC$\geq$ 0 or $\leq$ 0 were considered statistically significant. Statistically significant genes were compared with the Venny Program (https://bioinfogp.cnb.csic.es/tools/venny/) to find common DEGs and further analyses were continued with selected genes.

Functional analyses of DEGs

DAVID (https://david.ncifcrf.gov/) online tool was used to analyze the molecular pathways and biological processes in which common DEGs play a role in the dataset. Gene Ontology (GO) was determined according to biological processes (BP), cellular components (CC) and molecular functions (MF). Biological pathways have been identified with the Kyoto Encyclopedia of Genes and Genomes (KEGG). According to the function enrichment analysis, terms with P<0.05 were considered statistically significant.

Protein interaction analyses

First, common DEGs were imported into the STRING database (https://string-db.org/) to analyze protein-protein interactions (PPI) and protein interaction network was constructed with confidence limit >0.4. Network visualization and analysis of the topological properties of the network were performed using the cytohubba application in Cytoscape software (Cytoscape v3.9.2) and hub genes were detected. The selected proteins were the hub genes with the most interactions in the protein interaction network. Protein clusters in highly interconnected regions were demonstrated via MCODE analysis.

Data validation

Cell culture

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Human gingival fibroblasts (Accegen Biotechnology, Fairfield, New Jersey, USA, Cat no: ABC-TC3627) were incubated in high glucose conditions to mimic T2DM in vitro, as described previously.[16] After 48 hours of incubation, cells were detached from the culture plate for protein quantification. Total cell extracts were obtained using a tissue lysis buffer (Invitrogen, Carlsbad, CA, USA) containing 2% protease inhibitor cocktail. Within the top 40 genes with the highest score according to STRING analysis, TNF-α and Forkhead box protein A1 (FOXA1) were selected for data validation.

Protein quantification

For each gene, protein levels were compared between control and high glucose groups via ELISA assay. Human TNF-α ELISA Kit was purchased from Elabscience Biotechnology (Houston, TEX, USA) and human FOXA1 ELISA Kit was purchased from MyBioSource.com Inc. (San Diego, CA, USA). For this purpose, 100 μl of standard or samples were added to each well in a 96-well plate and incubated for 90 minutes at 37°C. After the removal of the liquid a 1:100 dilution of the biotinylated antibody (Ab) was added to the wells and incubated for 60 minutes at 37°C. Horseradish peroxidase (HRP) and substrate reagents were added one by one to visualize the Ab conjugates. The optical density (OD) value was detected at 450 nm. Results were shown as mean and standard deviation for three experimental replicates. Normally distributed data were analyzed with one-way ANOVA (Analysis of Variance) and non-parametric analysis were performed with Sidak's multiple comparisons test via GraphPad Prism Software 9.0.0. Significance level was set to P<0.05.

Results

Identification of common DEGs

The bidirectional relationship between diabetes and periodontitis allows both diseases to be controlled at the same time. Therefore, to understand the alterations that are only related to pathological conditions, common genes for poorly controlled and well-controlled groups were excluded from the study. Data sets were distinguished via GEO2R analysis according to the P and log2FC values (P<0.05 and log2FC$\geq$0 or $\leq$0). A total of 303 up- and downregulated common genes (185 and 118, respectively) for poorly controlled and well-controlled T2DM were excluded (Figure 1). 1008 genes were upregulated, while 610 genes were downregulated in periodontitis patients when T2DM was poorly controlled.

Functional analyses of common DEGs

Pathway enrichment analysis for DEGs were performed using DAVID software. DEGs with increasing and decreasing expression were found to be highly enriched in the 'Molecular Function (MF) group, followed by 'Biological Process (BP)' and 'Cellular Component (CC)' groups (P<0.05). Number of genes were elevated the most in the metal ion binding, regulation of transcription from RNA polymerase II promoter and ATP binding subgroups. GO terms and the list of important enriched genes are shown in Table 1.

When alterations in molecular pathways were analyzed via KEGG, it was demonstrated that downregulated genes were clustered in 9 pathways; two of the most altered ones were found to be

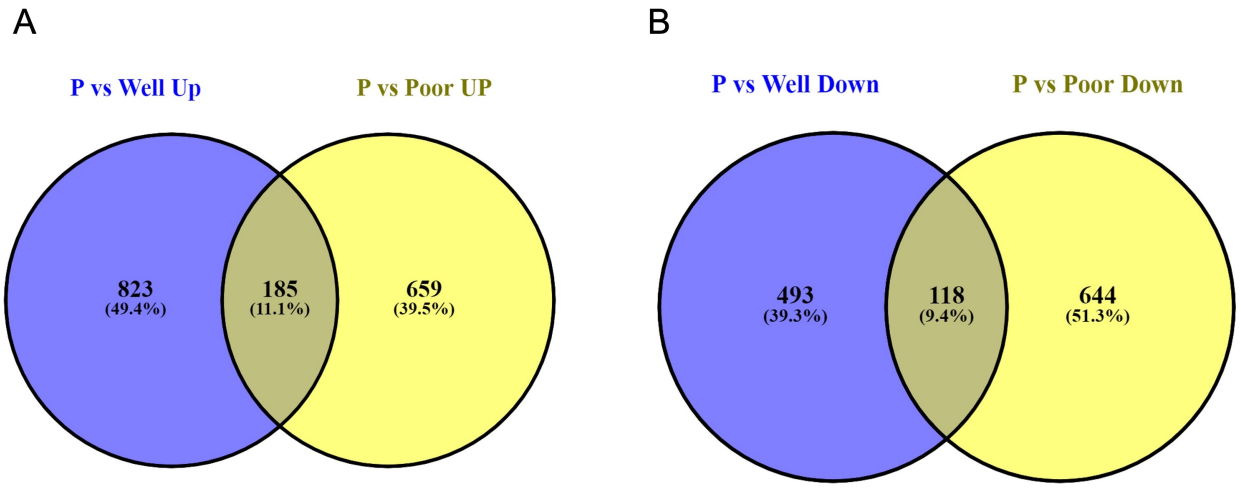

Figure 1. Venny diagram of GEO datasets for T2DM-poor and T2DM-well groups compared to the controls (P) (P<0.05). **A)** Upregulated genes. **B)** Downregulated genes. Common DEGs were excluded from the study. Further analyses were continued with T2DM-poor and P groups.

the cancer pathways and PI3K/Akt signaling pathway. Upregulated genes were enriched in 4 pathways including purine metabolism, parathyroid hormone metabolism, cGMP/PKG signaling pathway and Rap1 signaling pathway (Table 2).

Identification of PPI and Hub Genes

Integrated PPI Network for increasing and decreasing genes were analyzed using the STRING database. The gene interaction network involved 20 nodes indicating each DEG and 54 edges indicating interactions between defined nodes for increasing genes. Decreasing genes involved 487 nodes and 1368 edges (P<0.05). Following the STRING analysis, network analyzer in Cytoscape software was used to rank core genes according to their predicted scores. Hub genes were selected via cytoHubba analysis. Figure 2 shows the 20 genes with the highest score for both increasing and decreasing gene expressions. The score decreases from red to yellow. Hub nodes with highest score were SMAD4, HNF4A, SMARCA4 and SRC, TNF, RFC2, RFC3 genes with increasing and decreasing expression profiles, respectively. GO analysis revealed that SMAD4, HNF4A, SMARCA4 were responsible for the regulation of transcription from RNA polymerase II promoter, while only SMAD4 and HNF4A were engaged in sequence-specific DNA binding. Increasing SMAD4 activity was also related to metal ion binding. In addition, SMARCA4 was found to be recruited in chromatin remodeling, regulation of G0 to G1 transition and regulation of nucleotide-excision repair.

Highly interconnected regions were demonstrated via MCODE analysis (Table 3). Cut off score was set to 4. Protein clusters with the highest score were SMARCA4, MT-ND4, MT-CO2, GATA2, ATP5C1, NDUFB6, CDX2, FOXA1, HNF4A, NDUFB2 and SMAD4 with increasing expression, while expressions of RFC3, BARD1, NBN, XRCC6, TIMELESS, RAD1, RFC2 and FANCI were decreased.

Data validation

For the validation of bioinformatic analyses, human gingival fibroblasts were incubated in low and high glucose conditions and alterations in levels of proteins represented by hub genes were controlled. As predicted TNF-α levels significantly increased by 2 folds in high glucose conditions (P=0.005), while FOXA1 levels were also increased (P=0.023) (Figure 3).

Discussion

Bidirectional relationship between periodontitis and T2DM has been emphasized by many studies,[2,17,18] yet genetic alterations have not been completely revealed. In this study, high HbA1c levels caused by poorly controlled T2DM were linked to the elevated levels of prominent genes and pathways in periodontitis, for a better understanding of the underlying molecular mechanisms. According to KEGG analysis, altered pathways with the highest impact were found as follows; increased expression of i) Purine metabolism ii) Parathyroid hormone synthesis, secretion, and action iii) cGMP/PKG signaling pathway iv) Rap1 signaling pathway and decreased expression of i) Pathways in cancer ii) Proteoglycans in cancer iii) PI3K/Akt signaling pathway.

Components of the purine metabolism pathways are also known as early biomarkers of diabetes as demonstrated by Barnes et al.[19] Recently, elevated blood uric acid levels, which is the final product of the purine degradation pathway, have been shown to be associated with periodontitis.[20,21] In this study, this common pathway has been demonstrated for the first time to be elevated via high HbA1c levels in poorly controlled T2DM patients with periodontitis. Within the 10 genes recruited in this pathway, only PDE3B was demonstrated as downregulated in healthy patients compared to the T2DM-well-P group,[14] in contrast with our results. In our study, 10 genes related to the purine metabolism pathway were upregulated in T2DM-poor patients compared to the P group, including PDE3B gene. Increased expression of NT5E, ADCY9, NME7, PDE3B, PDE6C, ENPP3, NTPCR, ADCY7, GART and PDE9A were suggested as early genetic markers for T2DM related periodontitis for the first time in literature.

In our study a total of 8 genes, NR4A2, AKAP13, ADCY9, MEF2D, ADCY7, RXRG, FGFR1 and PLD2, that are responsible for the synthesis, secretion and action of parathyroid hormone has been found to be increased in periodontitis patients with poorly controlled T2DM. In parallel, recent studies revealed that intermittent administration of parathyroid hormone enhances periodontal healing through decreased inflammation in vivo.[22] In diabetic rats, it was demonstrated that topical or discontinuous parathyroid hormone uptake declines alveolar bone loss in experimental periodontitis[23]. Supporting our data, NR4A2 was ranked in the top 200 DEGs identified by an integrated analysis of three microarray data sets by Guo et al[19]. On the contrary, one of the upregulated genes, AKAP13, was found to be downregulated in symptomatic and asymptomatic periodon-

Table 1. GO analysis of altered genes in periodontitis patients with poorly controlled T2DM.

Upregulated Genes		
Category	**Term**	**Count**
Biological Process	GO:0000012~single strand break repair	6
	GO:0006357~regulation of transcription from RNA polymerase II promoter	67
	GO:0006338~chromatin remodeling	11
	GO:0070316~regulation of G0 to G1 transition	4
	GO:0006260~DNA replication	8
	GO:2000819~regulation of nucleotide-excision repair	4
Cellular Component	GO:0000785~chromatin	40
	GO:0016363~nuclear matrix	8
Molecular Function	GO:0003684~damaged DNA binding	9
	GO:0043565~sequence-specific DNA binding	18
	GO:1990837~sequence-specific double-stranded DNA binding	24
	GO:0046872~metal ion binding	87
	GO:0003700~transcription factor activity, sequence-specific DNA binding	23
Downregulated Genes		
Biological Process	GO:0045087~innate immune response	28
Cellular Component	GO:0035580~specific granule lumen	9
	GO:0031012~extracellular matrix	17
Molecular Function	GO:0003779~actin binding	21
	GO:0005525~GTP binding	21
	GO:0051015~actin filament binding	14
	GO:0003924~GTPase activity	16

Table 2. KEGG pathways of altered genes in periodontitis patients with poorly controlled T2DM.

KEGG Pathway	**Count**	**Upregulated Genes**
hsa00230:Purine metabolism	10	NT5E, ADCY9, NME7, PDE3B, PDE6C, ENPP3, NTPCR, ADCY7, GART, PDE9A
hsa04928:Parathyroid hormone synthesis, secretion and action	8	NR4A2, AKAP13, ADCY9, MEF2D, ADCY7, RXRG, FGFR1, PLD2
hsa04022:cGMP/PKG signaling pathway	10	NPPB, ADCY9, ROCK1, PDE3B, IRS2, ATP2B1, MEF2D, ADCY7, MYLK4, INS
hsa04015:Rap1 signaling pathway	11	ADCY9, SIPA1L1, FGF9, PARD6G, ARAP3, ADCY7, RAPGEF6, SIPA1, PFN2, FGFR1, INS
KEGG Pathway	**Count**	**Downregulated Genes**
hsa04810:Regulation of actin cytoskeleton	17	ACTR3, ACTR2, SRC, RDX, ITGA1, LPAR1, F2, MYL12A, MYLK, CDC42, FGF5, DIAPH2, ITGAD, CFL2, KRAS, EZR, SOS1
hsa05205:Proteoglycans in cancer	15	CAMK2D, SRC, RDX, ITPR2, FRS2, THBS1, TNF, ANK1, CDC42, CTTN, PLAU, ERBB4, KRAS, EZR, SOS1
hsa04621:NOD-like receptor signaling pathway	14	STAT1, DEFA4, ITPR2, TNF, ATG12, GPRC6A, TRAF3, TXNIP, MFN2, TRPM7, GBP1, CAMP, BIRC3, IFNAR1
hsa04530:Tight junction	13	ACTR3, ACTR2, HSPA4, SRC, RDX, MYL6B, MYL12A, CDC42, CTTN, CLDN14, PARD3, EZR, CRB3
hsa04611:Platelet activation	10	VWF, SRC, ADCY3, ITPR2, F2, MYL12A, PRKG1, PIK3CG, MYLK, PTGS1
hsa05417:Lipid and atherosclerosis	13	CAMK2D, HSPA4, SRC, EIF2S1, TNF, HSPD1, CDC42, CYP2C8, CYP2B6, TRAF3, KRAS, JAK2, BID
hsa04062:Chemokine signaling pathway	12	CDC42, CCL23, STAT1, SRC, PARD3, ADCY3, KRAS, CCR6, JAK2, SOS1, PIK3CG, PF4
hsa05200:Pathways in cancer	25	CAMK2D, LPAR1, ADCY3, LAMC1, STK4, FGF5, CDC42, BCL2L11, CCND2, CTNNA1, BAK1, JAK2, BID, RUNX1T1, ARHGEF11, EDN1, STAT1, F2, COL4A1, TRAF3, CCDC6, KRAS, SOS1, BIRC3, IFNAR1
hsa04151:PI3K/Akt signaling pathway	18	VWF, ITGA1, LPAR1, LAMC1, THBS1, EFNA4, PIK3CG, FGF5, RPTOR, BCL2L11, CCND2, COL4A1, ERBB4, KRAS, JAK2, SOS1, SGK2, IFNAR1

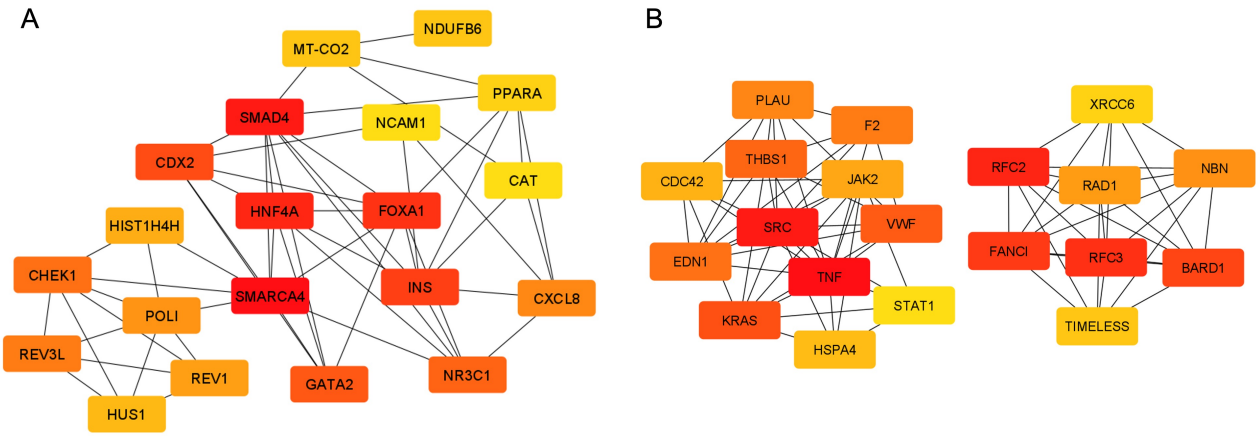

Figure 2. Top 20 genes for **A)** Upregulated genes. **B)** Downregulated genes within the identified PPI network according to STRING analysis (P<0.05).

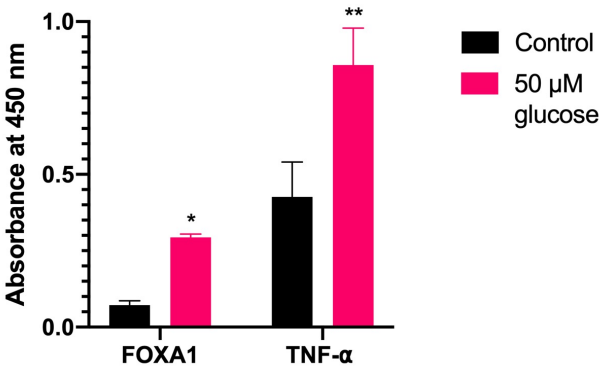

Figure 3. Results of ELISA assay for TNF-α and FOXA1 proteins in gingival fibroblasts treated with 50 µM high-glucose versus controls. Both FOXA1 and TNF-α levels were significantly altered as predicted. *P<0.05, **P<0.005

Table 3. MCODE analysis for protein clusters in the identified PPI network.

Score	Nodes	Edges	Upregulated Genes
5,2	11	26	SMARCA4, MT-ND4, MT-CO2, GATA2, ATP5C1, NDUFB6, CDX2, FOXA1, HNF4A, NDUFB2, SMAD4
5	5	10	POLK, HUS1, POLI, REV3L, REV1
4	4	6	VAPA, SNAP23, STX3, SYT9
4	4	6	HIBADH, GCDH, ALDH6A1, ACADSB
Score	**Nodes**	**Edges**	**Downregulated Genes**
7,714	8	27	RFC3, BARD1, NBN, XRCC6, TIMELESS, RAD1, RFC2, FANCI
6	6	15	PLAT, F2, EDN1, PLAU, THBS1, VWF
4,065	32	63	CEACAM8, CD5, KDM7A, MMP8, TNF, GRIN2A, PCDH10, JAK2, NEXN, CNTNAP2, CTNNA1, SOX2, COL4A1, CTTN, ITGA1, CFL2, CCND2, FCGR1A, SPTBN5, MYL12A, TNS1, EZR, IFNAR1, SCN1A, OLFM4, MYLK, RPTOR, COL5A1, BCL2L11, PIK3CG, TPM1, NRXN1
4	16	30	PSMC5, ERBB4, RAN, MUC1, CD24, HSPD1, MPO, CD274, CAMP, DEFA4, PSMC6, LTF, STAT1, EIF2S1, SOS1, PSMA4
4	26	50	EPB41L2, SFPQ, CDC42, RHOBTB1, STK4, EXOC7, CSTF2, TEAD1, SAV1, MAP4K4, DIAPH2, FRMD1, CLIC5, STXBP5, XPO5, CAMK2D, LAMC1, SRC, KRAS, AGO4, CLIC4, RALA, EXOC8, ACTR2, SF3B1, RDX
4	4	6	NOP9, WDR36, DDX52, TSR1

titis patients.[24] As a result, parathyroid hormone synthesis might be upregulated for regenerative purposes due to damage caused by high HbA1c levels in diabetic periodontitis patients. Supporting our hypothesis, FGFR1, also recruited in this particular pathway, was demonstrated as one of the regulators of periodontal regeneration in vivo and in vitro.[25,26] Among the upregulated pathways cGMP/PKG signaling pathway was found to be hypo-methylated in a comprehensive analysis of DNA methylation for periodontitis.[27] Rap1 signaling pathway was also ranked as an important elevated pathway for chronic periodontitis patients in a microarray study in which differentially expressed mRNAs were identified.[13] KEGG analysis in diabetic patients with periodontitis has also revealed an upregulation in Rap1 signaling pathway in a recent study by Kang et al.[15] In our study a total of 21 genes were upregulated for cGMP/PKG and RAP1 signaling pathways. The PI3K/Akt signaling pathway is one of the most common activated pathways associated with human cancer.[28] In periodontitis, associated species are known to induce Toll-like receptors (TLRs) and antiapoptotic pathways (JAK/STAT, MAPK etc.), including PI3K/Akt pathway.[29] In presence of several chemical agents, PI3K/Akt pathway is also known for induction of osteogenic differentiation[30,31] and migration in periodontal ligament cells[32], as well as inflammation of gingival fibroblasts in vitro.[33,34] In our study, a total of 40 genes in cancer pathways and proteoglycans in cancer pathway were downregulated parallel to 18 genes in PI3K/Akt signaling pathway, indicating a possible relationship between poor control of diabetes in chronic periodontitis and oral cancers.

Besides altered common pathways, a total of 40 up- and down-regulated hub genes were demonstrated according to STRING analysis. Among these genes, TNF is well-known to be associated with both periodontitis and T2DM enhancing a 2-way relationship.[35] TNF-α, a member of the TNF family, is produced due to periodontal inflammation increasing insulin sensitivity, which triggers adipocytes for more TNF-α production increasing the risk factors for periodontitis.[35] TNF protein family also plays an important role in the NOD-like receptor signaling pathway which has been demonstrated within the downregulated pathways in our study according to KEGG analysis. Comparably, various studies also reported alterations in NOD-like receptor signaling pathway in periodontitis patients,[36–38] as well as TNF.[11,14] In this study, TNF-α was chosen for data validation together with another hub gene, FOXA1, that have not been reported to be associated with neither periodontitis nor diabetes yet. ELISA assay revealed significant alterations in both proteins as projected.

Conclusion

In this study, alterations in a total of 13 pathways and 40 hub genes were demonstrated for periodontitis patients with poorly controlled T2DM via GO, KEGG and STRING analyses. MCODE analysis revealed 116 genes in 10 protein clusters within the defined PPI network. Although bioinformatic analyses were confirmed via ELISA assay for two hub genes, protein-protein interactions may differ in in vivo conditions. Therefore, further validation of the presented data is needed in animal and human studies in vivo, as well as in a periodontitis model in vitro.

Author Contributions

Study design; DAT, AKG, literature review; DAT, AKG, data collection and processing; AKG statistics; AKG, preparation of the manuscript; DAT, AKG.

Conflict of Interest

The authors declare no conflict of interest.

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