CXCR4 and RAC2 as Biomarkers of Periodontitis and Their Potential Influence on Macrophage Infiltration

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Abstract

Objective Chronic periodontitis is an incurable infectious disease characterized by complex inflammatory immune response. This study aimed was to screen and identify effective biomarkers correlated with macrophage in chronic periodontitis.

Materials and methods Periodontitis datasets obtained from the gene expression omnibus (GEO) were analyzed to identify differentially expressed genes (DEGs). Functional enrichment analysis of DEGs by GO and KEGG revealed multiple biological processes and signaling pathways related to immune response. Further analysis was performed on two immune-related gene databases, ImmPort and innateDB, to identify DEGs related to immune cells infiltration in chronic periodontitis. Subsequently, Protein-protein interaction (PPI) network were established to reveal hub genes with MCC algorithm using CytoHubba tool. Enrichment analysis in DisGeNet showed that immune-related DEGs were enriched in periodontitis. Next, CIBERSORTx tool was analyzed to investigate immune cells infiltration in GSE10334 dataset.

Results The results demonstrated differential macrophage infiltration in periodontitis. Notably, RAC2 and CXCR4 were identified as biomarkers correlated with M2 macrophages in chronic periodontitis. Finally, receiver operating curve (ROC) was plotted to determine the diagnostic value of identified hub genes which was then validated using relevant datasets of macrophages.

Conclusion RAC2 and CXCR4 are potential biomarker of chronic periodontitis correlated with M2 macrophages infiltration. Further experiments are needed to more deeply explore the roles of CXCR4 and RAC2 in the molecular mechanism of M2 macrophages in periodontitis microenvironment.

Background

Chronic periodontitis (CP) is a disease caused by inflammatory-induced destruction of the periodontium. Specifically, it stems from inflammation of the supportive tissue surrounding teeth, affecting the gingiva, periodontal ligament, and alveolar bone [1]. Based on radiographic examination, periodontitis presents with loss of alveolar bone around teeth, and this is considered irreversible [2]. periodontitis affects about 10-15% of the world's population and is the major cause of tooth loss in adults [3]. The available treatments for periodontitis are far from being satisfactory [4, 5]. Evidence suggests that periodontitis is rather a complex disease caused by multiple factors complexity that interact with the subgingival plaque, host's own immunity, and inflammatory responses [6]. However, the mechanisms driving such interactions are not clearly defined [7]. Therefore, understanding these mechanism may reveal avenues for the prevention and treatment of periodontitis and more accurate biomarker to assess and monitor disease risk [8].

A large number of previous studies have confirmed that immune cell infiltration plays an important role in the occurrence and pathogenesis of chronic periodontitis [9, 10]. In the past few decades, several studies have uncovered that the components of immune response involved in the pathogenesis of periodontitis

[11, 12]. Elsewhere, bacterial products were found to activate the host immune system, and B cells were implicated in the occurrence of periodontitis lesions [13, 14]. *P. gingivalis*, a strain that drives immunopathogenesis of chronic periodontitis, expresses diverse virulence factors through which it evades the host's innate immunity and suppresses the host's adaptive immunity [15, 16]. *P. gingivalis* that invades gingival epithelial cells can escape the host's immunity system, and indirectly regulates the function of immune cell [17, 18]. In addition, T-cells and B-cells are involved in the pathogenesis of periodontal disease [19, 20]. This implies that evaluating the infiltration level of immune cells reveal the molecular mechanisms driving chronic periodontitis and promoting the development of novel immunotherapeutic agents for management of this disease.

This study aimed to identify biomarkers related to immune cells using bioinformatics tools. In recent years, it has been reported that application of transcriptomic technologies can potentially help to understand disease mechanisms and discover disease biomarkers [21, 22]. Indeed, numerous studies have screened important biomarkers of chronic periodontitis by analyzing public databases GEO database [23–27]. However, none of the aforementioned studies investigated the relationship between immune cells infiltration and differentially gene expression in chronic periodontitis.

Methods

2.1. Retrieval of microarray data

Three gene expression profile datasets: GSE10334 [28], GSE16134 [29], and GSE23586 [30] were retrieved from Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>) database [31] and used for analysis of DEGs in chronic periodontitis. Detailed information of the datasets used in this study is presented in Table 1. All experimental protocol were approved by Liaocheng People's hospital and ethical approval was provided by the ethical committee of Liaocheng People's hospital. Immune related genes were retrieved from innateDB [31] and ImmPort [32] databases. Validation of selected genes was performed using two datasets for macrophages (GSE24897).

Table 1
Details for GEO Datasets in this study

Accession	Platform	Tissue	Samples (H:P)	Year
GSE10334	GPL570	Gingival	64:183	2008
GSE16134	GPL570	Gingival	69:241	2009
GSE23586	GPL570	Gingival	3:3	2010
GSE24897	GPL570	human macrophages	3:3:3:3	2010

2.2. Preprocessing of data and screening of DEGs

“GEOquery” package in R software (v4.1) (Download R-4.1.0 for Windows) was used to retrieve the expression profile of microarray datasets (GSE10334, GSE16134, and GSE23586) from GEO database. Range migration algorithm (RMA) was used to perform background correction and for normalization of data. Expression profiles of microarray datasets were analyzed using “limma” package [33]. Genes with $P < 0.05$ and $|\log_2FC| > 1.5$ were considered DEGs. Overlapping DEGs were visualized using Venn diagram. Overlapping upregulated genes and downregulated genes were then identified.

2.3. Functional and pathway enrichment analysis

Gene ontology (GO) functional analysis and Kyoto encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed using “clusterProfiler” package in R software based on DEGs to identify terms and pathways that were enriched. GO terms and KEGG pathways with $p < 0.05$ were considered significantly enriched.

2.4. Identification and functional enrichment analysis of immune-related DEGs

Two immune-related gene (IRG) datasets were retrieved from ImmPort database (<https://www.immport.org/shared/genelists>) and innateDB database (<https://www.innatedb.ca>). Differentially expressed immune-related genes (DEIRGs) were identified by intersecting IRG datasets and DEGs. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (string-db.org) was then used for construction of a PPI network of DEIRGs with a confidence score > 0.4 . the PPI network was visualized using Cytoscape software (version 3.8.1). Enrichment analysis of the 20 DEIRGs associated with periodontitis was performed using DisGeNET tool (<http://www.disgenet.org/>). Further, a network of the enriched terms of immune-related DEGs was constructed using ColorByCluster tool.

2.5. Analysis of immune infiltration in chronic periodontitis

Gene expression matrix data (GSE10334) were uploaded to “CIBERSORTx” website [34] to obtain the immune cell infiltration matrix to explore the role of immune infiltration in chronic periodontitis. An immune cell abundance map and Box diagrams were generated using “ggplot2” package to visualize differences in immune cell infiltration and the proportion of 4 aggregated immune cell types. Spearman correlation analysis was performed to explore the potential relationship between IREGs and infiltrating macrophages. The correlation between IREGs and infiltrating macrophages was then visualized using “ggplot2” package in R.

2.6. Validation and Prognostic value of immune-related genes

A receiver operating characteristic (ROC) curve was generated to explore the prognostic value of selected IREGs that correlated with macrophages in chronic periodontitis. The prognostic value of the selected genes was then validated using GSE24897 [35] macrophages datasets.

2.7 Statistical analysis

Statistical analyses in the current study were performed in R software v4.1. Differences between two groups were determined using t-test. Differences in expression level of macrophages in healthy and periodontitis groups were determined using Wilcoxon's test. Spearman correlation analysis was performed to explore the correlation between IRGs and immune cells infiltration. Prognostic values of IREGs were evaluated by ROC analysis using the "pROC" R package. $p < 0.05$ was considered statistically significant.

Results

3.1 DEGs were identified between healthy and periodontitis groups

The experimental design of the current study is presented in Fig. 1. Analysis showed that 203 genes were significantly upregulated in the three datasets (338 in GSE10334; 439 in GSE16134; and 1595 in GSE23585), whereas 135 genes were significantly downregulated (280 in GSE10334; 251 in GSE16134; and 664 in GSE23585) (Fig. 2A and B).

3.2 Go And Kegg Functional Enrichment Analysis

GO functional annotation and KEGG pathway enrichment analysis of the 338 DEGs were performed using "clusterProfiler" and "pathview" R packages. The findings on significantly enriched terms and pathways are presented in Fig. 2C. Biological process (BP) analysis of DEGs showed that cell chemotaxis, cornification epidermis development, leukocyte chemotaxis, myeloid leukocyte migration, neutrophil activation involved in immune response, neutrophil degranulation, phagocytosis, and T cell activation were the significantly enriched BP terms. Findings from Molecular function (MF) analysis indicated that chemokine activity, cytokine activity, cytokine binding, cytokine receptor activity, growth factor binding, growth factor receptor binding, IgG binding, immune receptor activity, immunoglobulin binding, and integrin binding were the significantly enriched MF terms. In addition, cellular component (CC) analysis showed that DEGs were mainly implicated in actin filament, collagen-containing extracellular matrix, cornified envelope, desmosome, external side of plasma membrane, ficolin-1-rich granule, ficolin-1-rich granule lumen, ficolin-1-rich granule membrane, secretory granule membrane, and tertiary granule. KEGG pathway analysis showed that the DEGs were significantly enriched in chemokine signaling pathway, phagosome, leukocyte transendothelial migration, malaria, staphylococcus aureus infection and natural killer cell mediated cytotoxicity (Fig. 2D). BP terms associated with immune response were further explore (Fig. 2E).

3.3 Functional enrichment analysis of immune-related differentially expressed genes.

A total of 20 immune related genes were identified (Fig. 3A). A PPI network for the 20 immune related genes presented in Fig. 3B. Enrichment analysis in DisGeNET showed that Periodontitis was the tenth

enriched term (Fig. 3C). Analysis of the 20 immune related genes using ColorByCluster showed that they are mainly implicated in inflammatory immune response (Fig. 3D).

3.4 Immune Cell Infiltration Results

Distribution of the 22 types of immune cells in GSE10334 dataset is presented in Fig. 4A. Analysis of immune cell infiltration showed that, plasma cells, M2 macrophages, CD8 T cells, CD4 memory resting T cells, neutrophils, resting mast cells, memory B cells, follicular helper T cells, activated dendritic cells, M1 macrophages, CD4 memory activated T cells, naïve B cells were significantly infiltrated in chronic periodontitis samples compared with the levels in the healthy control samples. Further, immune cell composition (22 immune cell types and 4 aggregate immune cell types) in the periodontitis tissue was explore using CIBERSORTx algorithm. The 4 aggregated immune cell types were total lymphocytes, total macrophage (M0, M1, and M2 macrophage percentages), total dendritic cell (activated and resting dendritic cell percentages), and total mast cells (activated and resting mast cell percentages). The results showed that total lymphocytes were significantly higher in the periodontitis group compared with the level in the health group. Analysis showed that total dendritic cells, total macrophages, and total mast cells were significantly lower in periodontitis group compared with the level in the health group (Fig. 4B). these findings showed that M1 and M2 macrophage fraction was lower in periodontitis compared with the level in healthy gingival tissue (Fig. 4C and D). Fractions of M0 macrophages were not significantly different between periodontitis and health tissues (Fig. 4E). Analysis showed that M2/M1 macrophage ratio was lower ($p<0.037$) in periodontitis compared with the ration in healthy gingival tissue (Fig. 4F).

3.5 Correlation between the IREGs and macrophages in periodontitis group

The findings from the above sections indicate that macrophages are implicated in progression of periodontitis. Therefore, further analysis was conducted to explore the relationship between the IREGs and macrophages in the periodontitis group. The top two ranked results by correlation coefficient were CXCR4 and RAC2. The results showed that RAC2 was positively correlated with M2 macrophage ($r=-0.35$, $p=1.2e-08$) (Fig. 5A); CXCR4 was positively correlated with M2 macrophage ($r=-0.36$, $p=4.4e-09$) (Fig. 5B). ROC curve was established to explore the diagnostic significance of CXCR4 and RAC2 in chronic periodontitis (Fig. 5C and D). Details on ROC analysis results are presented in Table 2.

Table 2
Receive operating characteristic (ROC) analysis of
selected genes.

Index (%) dataset		CXCR4	RAC2
AUC	GSE10334	86.6205	85.0324
	GSE16134	88.6643	87.8465
Sensitivity	GSE10334	85.7923	89.0710
	GSE16134	80.0829	89.6265
Specificity	GSE10334	75	71.8750
	GSE16134	85.5072	75.3623
Predictive Value	GSE10334	90.7514	90.0552
	GSE16134	95.0738	92.7038

3.7 Screening and validation of the two biomarkers correlated with macrophages in Periodontitis

CXCR4 and RAC2 showed significant correlation with macrophages and high diagnostic value in periodontitis. The expression levels of CXCR4 and RAC2 were validated using human macrophages treated with *P. gingivalis* and its components (GSE24897) (Fig. 5E-F). The findings showed that expression levels of CXCR4 and RAC2 in the experimental groups (Group PG, Group LFG, and Group CG) were significantly different compared with of the level in the control (saline group).

Discussion

The findings for the current study indicated that CXCR4 and RAC2 may regulate the role of macrophages in gingival tissues with periodontitis. Notably, the experimental conditions for *P. gingivalis* for stimulation of macrophages do not reflect the *in vivo* conditions, and thus further genetic and phenotypic changes should be explored *in vivo*.

Previous studies have reported that neutrophils, T cells, and leukocytes are implicated in the inflammatory response and immune escape process of periodontitis[36, 37]. Attachment, adhesion and retention of primary human gingival fibroblasts and microvascular formation are the main healing processes of periodontitis against microbial damage [38–41]. The level of serum IgG and salivary cross-reactive IgG in patients with periodontitis is significantly high and is correlated with the severity of periodontitis [42]. Autophagy mediated regulation results in changes in immune microenvironment of periodontitis [43]. Fcγ R-mediated phagocytosis stimulates local endocytosis of human neutrophils [44]. Findings from previous studies and the results from the current study indicate that immune response modulates the pathological process of periodontitis.

Immune landscape of periodontitis has been explored previously, and studies reported that resting dendritic cell, activated dendritic cell, and follicular T helper cells are key immune cells associated with periodontitis [45]. Analysis of the GSE10334 dataset showed similar finding on infiltration of immune cells, indicating that the findings from the current study are reliable. Differences in immune infiltrating cells between periodontitis gingival tissue and healthy gingival tissue were determine to further explore the role of immune cell infiltration in periodontitis.

Findings from animal experiments show that activation of hypoxia-inducible factor-1 α (HIF-1 α) decreases the ratio of M1/M2 macrophages in periodontal tissue, and downregulates the levels of pro-inflammatory cytokines, such as, TNF- α and IL-6, whereas the level of anti-inflammatory factors such as IL-10 and IL-4 is upregulated [46]. Gingival crevicular fluid (GCF) from patients with periodontitis can induce transformation of blood derived monocytes into destructive macrophages, thus changing the genotype and phenotype of periodontal resident cells. These changes aggravate periodontal damage, and limits periodontitis healing and inflammatory regression [47]. Further analysis was conducted to explore the molecular mechanisms of macrophages in progression of periodontitis.

CXCR4 and RAC2 are two top correlated with decreased M2 macrophages. Administration of AMD3100, a potent CXCR4 antagonist, inhibits periodontal bone loss induced by *P. gingivalis* [48]. CXCR4 signal transduction inhibits release of nitric oxide from infiltrating macrophages and is implicated in modulation of the mechanical sensitivity of periodontitis induced by *P. gingivalis* [49]. Previous studies reported that CXCR4, and RAC2 are implicated in immune response mechanisms of periodontitis by mediating the function of M2 macrophages. ROC curve analysis was performed to explore the diagnostic value of CXCR4 and RAC2 in periodontitis. Further analysis showed that CXCR4 and RAC2 were differently expressed in macrophages treated with *P. gingivalis* and its components for 2 hours and the effect lasted for 9 days. Although findings from previous studies are consistent with the findings of the current study, experimental studies should be conducted to explore the reliability and validate the findings from this study.

Conclusions

In summary, the findings showed that CXCR4 and RAC2 genes are significantly correlated with M2 macrophages as potential biomarkers in periodontitis. Further studies should be conducted on the selected genes associated with immune responses to determine targets of periodontitis immunotherapy and further improve immunomodulatory therapies for periodontitis patients.

Abbreviations

CXCR4	Chemokine CXC receptor 4
RAC2	Ras-related C3 botulinum toxin substrate 2
DEGs	Differentially expressed genes
GEO	Gene expression omnibus
GO	Gene ontology
KEGG	Kyoto encyclopedia of Genes and Genomes
PPI	Protein-protein interaction
ROC	Receiver operating curve
CP	Chronic periodontitis
PMNs	Polymorphonuclear leukocytes
TLR2	Toll-like receptor 2
MIF	Macrophage migration inhibitory factor
RMA	Range migration algorithm
IRG	Immune-related gene
DEIRGs	Differentially expressed immune-related genes
BP	Biological process
MF	Molecular function
CC	Cellular component

Declarations

Ethics approval and consent to participate

All research procedures were approved by the ethics committee of Liaocheng People's Hospital (2022001).

Consent for publication

This manuscript does not involve details, images, or videos relating to individual participants.

Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DW. W and YZ. C contributed equally to this work, DW. W and YZ. C were responsible for study concept and design, J.Z and D.S were involved in acquisition of data, Analysis and interpretation of data was performed by XF. T and J.W. XF. T wrote and edited the manuscript. All authors contributed to the article and approved the submitted manuscript.

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Figures

Figure 1

A flow diagram showing experimental design of current study.

Figure 2

Identification of differentially expressed genes (DEGs) in chronic periodontitis and functional enrichment analysis of DEGs. **(A)** Upregulated DEGs. **(B)** Downregulated DEGs; **(C)** Gene ontology (GO) enrichment analysis. The vertical axis represents the number of DEGs under the GO term. **(D)** KEGG pathway analysis of DEGs. **(E)** Chord plot showing the relationship between DEGs and immune related GO biological processes terms.

Figure 3

Functional enrichment analysis of immune-related differentially expressed genes. **(A)** Venn diagrams showing the numbers of overlapping genes among DEGs, innateDB and ImmPort datasets. **(B)** Protein-protein interaction (PPI) network of immune related DEGs. Eight hub genes were identified in the densest connected regions by MCC algorithm using CytoHubba plugin. Scores are indicated in different colors. **(C)** Summary of enrichment analysis using DisGeNET tool. Horizontal axis represents the number of immune

related DEGs using DisGeNET. The tenth enrichment result was Periodontitis. **(D)** Network of enriched terms of immune related DEGs, showing the top 20 terms. Each cluster ID is indicated with a different color.

Figure 4
Analysis and visualization of immune cell infiltration. **(A)** Proportion of the 22 types of immune cells. **(B)** Proportion of 4 aggregated immune cell types. **(C)** Macrophage subfractions in chronic periodontitis

gingival tissue. Each dot represents one patient. Mean values and standard deviations for each cell subset including M1 macrophage, M2 macrophage, M0 macrophage, and M2/M1 ratio.

Figure 5

Relationship between expression of immune related hub genes in periodontitis group and M2 macrophage. (A-B) Scatter plot showing the correlation between expression level of RAC2 and CXCR4 genes and M2 macrophage levels. ROC plot and validation of selected genes using *in vitro* assays. (C-D) Diagnostic value of selected hub genes was determined by ROC curve analysis using GSE10334 and GSE16134 dataset. (E-F) Expression level of CXCR4 and RAC2 genes in GSE24897 dataset

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GSE10334normalization.pdf](#)
- [GSE16134normalization.pdf](#)
- [GSE23586normalization.pdf](#)