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Comprehensive Analysis of Immune Infiltration and Key Genes in Peri-Implantitis Using Bioinformatics and Molecular Biology Approaches

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCE 1,2 Qingxun Meng*
AEFG 1,2 Jing Han*
CD 1,2 Xi Zhang
BCF 1,2 Wenxuan Su
BC 1,2 Beibei Liu
BC 1,2 Taicheng Liu

1 Tianjin Key Laboratory of Oral and Maxillofacial Function Reconstruction, Tianjin Stomatological Hospital, The Affiliated Stomatological Hospital of Nankai University, Tianjin, PR China
2 School of Medicine, Nankai University, Tianjin, PR China

* Qingxun Meng and Jing Han have contributed equally to this work

Corresponding Author: Jing Han, e-mail: hanj0203@163.com

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Conflict of interest: None declared

Background: Peri-implantitis is the main cause of failure of implant treatment, and there is little research on its molecular mechanism. This study aimed to identify key biomarkers and immune infiltration of peri-implantitis using a bioinformatics method.

Material/Methods: Three Gene Ontology (GO) gene expression profiles were selected from the Gene Expression Omnibus. Differentially expressed genes (DEGs) were identified by the LIMMA package, and functional correlations of DEGs were analyzed by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis. Information on immune-related genes was obtained from ImmPort (https://www.immport.org) and InnateDB (http://www.innatedb.com). Immune-related DEGs were screened by least absolute shrinkage and selection operator (LASSO) and support vector machine-recursive feature elimination (SVM-RFE). The single-sample Gene Set Enrichment Analysis algorithm was used to analyze immune cell infiltration in gingival tissue between peri-implantitis and normal controls. Finally, results of bioinformatics analysis were verified by qPCR.

Results: A total of 398 DEGs were identified, of which 96 were immune-related. Enrichment analysis showed these genes were enriched in inflammatory response, leucocyte chemotaxis, immune response-regulating signaling pathway, and cell activation. Seven key genes were selected by LASSO and SVM-RFE. Receiver operating characteristic curve results showed these genes had excellent diagnostic efficacy. Results of qPCR showed significant differences in the expression of these genes.

Conclusions: Differences in key genes and immune infiltration between peri-implantitis and gingival tissues of normal controls may provide new insights into the development of peri-implantitis. Elucidating the difference in immune infiltration between peri-implantitis tissues and normal tissues will help to understand the development of peri-implantitis.

Keywords: Peri-Implantitis • Computational Biology

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Introduction

Implant restoration has gradually become the first choice for patients because of its excellent masticatory efficiency, excellent aesthetic effect, and maximum restoration of the function of natural teeth [1,2]. However, peri-implantitis seriously affects the service life of implants [3]. Peri-implantitis occurs in peri-implant tissues and is characterized by inflammation of the soft tissue around the implant and progressive loss of hard tissue [4]. The incidence of peri-implantitis is high, with an average incidence of 22% reported [5]. Plaque is considered to be the main inducing factor of peri-implantitis, and the treatment of plaque is also the main means to treat peri-implantitis at present; however, the clinical effect of mechanical plaque removal and antimicrobial treatment is not ideal [6,7]. According to a systematic review in the Cochrane database, the recurrence rate of peri-implantitis is even as high as 100% in the 1-year follow-up period [8]. Therefore, it is necessary to explore the molecular characteristics and mechanisms of the occurrence and development of peri-implantitis. Immune reaction plays an important role in the progression of peri-implantitis [9]. On one hand, immune reaction can remove pathogenic microorganisms, toxins, and other harmful substances; on the other hand, immune reaction can also directly or indirectly lead to bone resorption through the proliferation and activation of osteoclasts [10]. Although the critical role of immune reaction in the development of peri-implantitis has been established, the specific molecular mechanisms are still unclear. Related studies have confirmed that immune reactions are closely related to the occurrence and development of peri-implantitis [11-14]. Exploring immune-related genes in peri-implantitis is of great significance for the molecular diagnosis and immunotherapy of this condition.

Bioinformatics is an interdisciplinary subject combining molecular biology, computer science, artificial intelligence, and statistics. It can be used to analyze a large number of biological data and has been widely used to identify gene markers, which are valuable for disease diagnosis, treatment, and prognosis [15]. As an emerging technology, microarray analysis can efficiently collect biological information and widely collect expressed genes in healthy or disease tissues to analyze the interaction between molecules and the mechanism of regulating different biological states of health and disease by computer.

In this study, machine learning methods were used to identify immune-related gene and immune cell infiltration in peri-implantitis, which was verified in clinical samples.

Material and Methods

Peri-Implantitis Gene Data Sets and Sequencing Data

Three gene expression datasets of patients with peri-implantitis were downloaded from Gene Expression Omnibus (GEO), including GSE33774 [16], GSE57631 [17], and GSE106090 [18]. The GSE33774 dataset included 7 peri-implantitis tissue samples and 8 normal tissue samples. GSE57631 contained 6 peri-implantitis tissue samples and 2 normal tissue samples. GSE106090 contained 6 peri-implantitis tissue samples and 6 normal tissue samples. GSE33774 was sequenced by the GPL6244 platform, GSE57631 was sequenced by the GPL15034 platform, and GSE106090 was sequenced by the GPL21827 platform, and all samples were from humans. After removing the genes with missing values, the R package "sva" [19] was used to debatch the above 3 datasets. After standardized processing, a boxplot was used to show the differences before and after batch removal. We obtained 19 peri-implantitis samples and 16 normal tissue samples.

Immune-Related Differentially Expressed Gene Analysis

To analyze the differentially expressed genes (DEGs) in healthy tissues and peri-implantitis tissues, the "ggplot2" package was used to perform principal component analysis on the

Table 1. Primer sequences of related genes.

Gene	Primer sequences forward	Primer sequences reverse
FCGR3A	CCACTCCAGTGTGGCATCAT	TTGGGAGATCTTCAGTCCGC
CASP10	CCTTGGAGCACACAGAGGAT	GATTTCATGGCCAGCCTTCAG
XBP1	GAAGGCGCTGAGGAGGAAAC	ACTTGCTGTTCCAGCTCACT
C1QB	AGTAGGCTCTCGGCTCCTG	TGTCAGACGCCTCCTGGG
SERPINB9	ACTAGGTGGCAGGCCC	GCAGAGGAGATGCTCACAGG
LGR4	CTTCACCCAAGCGCTACAAT	CTCGAATGGCTTCACTGGGT
CHP2	ACAGTATTCGGCGAGAGACC	GGAGATCCATGCGGCTCAG
GAPDH	GTGGACCTGACCTGCCGTCTAG	GAGTGGGTGTCGCTGTTGAAGTC

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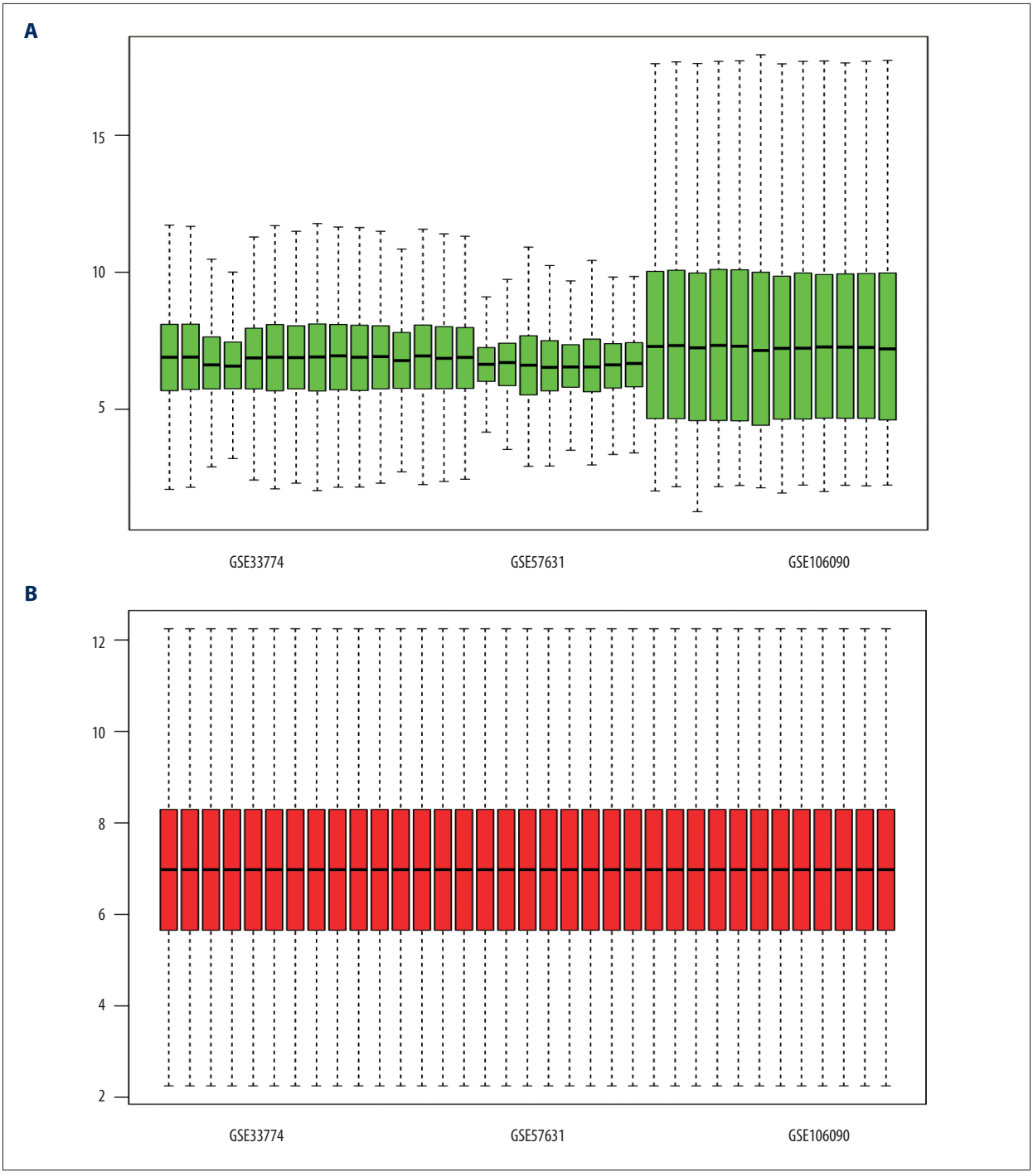

Figure 1. Standardization of datasets. (**A**) Data sets before standardization. (**B**) Datasets after standardization.

standardized datasets, and 2-dimensional principal component analysis maps were drawn to identify the differences between the normal group and disease group. DEG expression was identified using the Bayesian test. The log2 (fold change |FC|) >1 and *P* value <0.05 was considered statistically significant. The R packages "Pheatmap" and "ggplot2" were used to create heat maps and volcano maps for the DEGs. ImmPort (https://www.immport.org) [20] and InnateDB (http://www.innatedb.com) [21] are large biological information databases related to immunity, from which we obtained immune-related gene datasets, screened immune-related genes among the DEGs according to the datasets, and visualized the immune-related DEGs.

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Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis of Immune-Related Differentially Expressed Genes

Metascape (https://metascape.org/gp/index.html#/main/step1) [22] is a website tool that provides functional annotation for genes, with fast update speed and comprehensive data coverage. We imported immune-related DEGs into the Metascape platform for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis.

Least Absolute Shrinkage and Selection Operator Logistic Regression Model and Support Vector Machine-Recursive Feature Elimination Feature Selection Process to Screen Hub Genes

Least absolute shrinkage and selection operator (LASSO) logistic regression and support vector machine-recursive feature elimination (SVM-RFE) feature selection processes were used to find hub genes associated with peri-implantitis. We applied the LASSO algorithm in the R package "glmnet" [23]. SVM-RFE is a mechanical learning method based on a support vector machine [24]. The SVM model was constructed by R package "e1071" to further identify the key genes in peri-implantitis [25]. Finally, the intersection of genes screened by LASSO and SVM-RFE was used for subsequent analysis. $P<0.05$ was considered to be statistically significant. The receiver operating characteristic (ROC) curves of these genes were plotted by R package "pROC".

Immune Infiltration by Single-Sample Gene Set Enrichment Analysis

Single-sample Gene Set Enrichment Analysis (ssGSEA) can be used to quantify the infiltration level of a variety of immune cells, including T lymphocytes, dendritic cells, and natural killer cells [26]. The infiltration of 23 immune cells in the 3 datasets was assessed by ssGSEA. Subsequently, we conducted Spearman correlation analysis to reveal the correlation between key genes and immune cells. We used the R package "Pheatmap" to visualize the results.

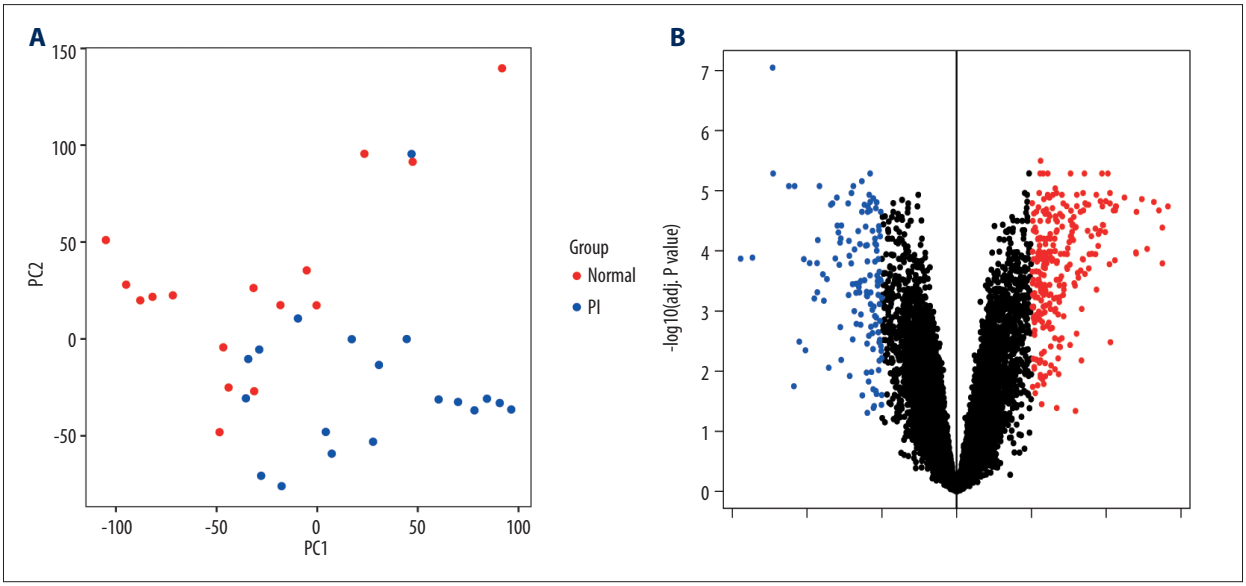
External Validation of Key Genes was Performed by qRT-PCR

This study was approved by the Medical Ethics Committee of Stomatological Hospital, Nankai University. To further verify the expression of hub genes, we obtained 6 human gingival samples from the peri-implantitis group and healthy group, respectively, according to the diagnostic criteria [27]. Total RNA in gingival samples was extracted with a Takara kit (Takara, Japan). Total RNA was reverse transcribed into cDNA using the Prime Script RT Reagent kit (Takara, Japan). The SYBR Premix Ex TaqII kit (TaKaRa, Japan) was used for qRT-PCR analysis, using the Roche Light Cycler 480 sequence detection system (Roche Diagnostics, Switzerland). The primer sequences of related genes are shown in **Table 1**. The relative expression levels of genes were calculated by the 2-ΔΔCt method. $P<0.05$ indicated a statistically significant difference.

Results

Identification of DEGs

Through the boxplot, we found that there was no obvious deviation between the standardized datasets (Figure 1), and these datasets could be used for the following analysis. The principle component analysis showed that the normal samples and



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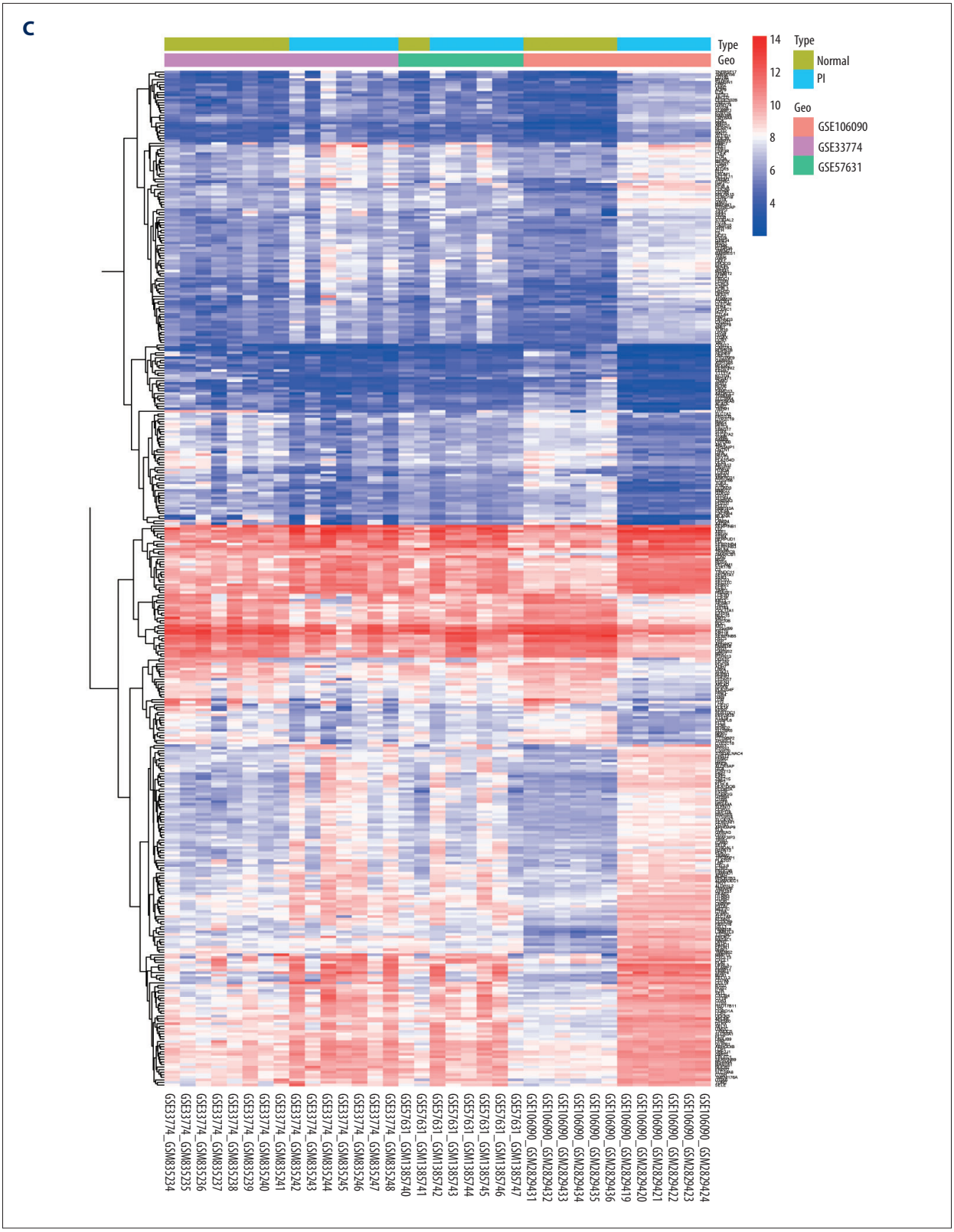

Figure 2. Differential expressed genes in peri-implantitis and healthy samples. (**A**) Principal component analysis for 3 datasets. (**B, C**) A volcano plot and heat map showing the 398 differentially expressed genes. Red indicates upregulated genes, and blue indicates downregulated genes.

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peri-implantitis samples were clustering significantly, indicating that the samples were from reliable sources, and there was a significant difference between the disease group and the healthy group (**Figure 2A**). After data processing, 398 DEGs were screened by the R package "LIMMA" (**Figure 2B, 2C**).

According to the immune gene datasets obtained from ImmPort and InnateD, 96 immune-related DEGs were screened out, and these immune-related genes that were differentially expressed in the peri-implantitis group and the normal group were demonstrated by a heat map and volcano map (**Figure 3A, 3B**).

Functional Enrichment Analysis

To enhance the understanding of the biological functions of 96 immune-related genes, we performed GO and KEGG enrichment analysis. The enrichment analysis results of Metascape for differentially expressed immune-related genes are shown in **Figure 4**.

The most significant enrichment terms included inflammatory response, leukocyte chemotaxis, immune response-regulating signaling pathway, and cell activation. The results of the analysis indicated that immune response plays an important role in peri-implantitis.

Genes Screened by LASSO Logistic Regression and SVM-RFE

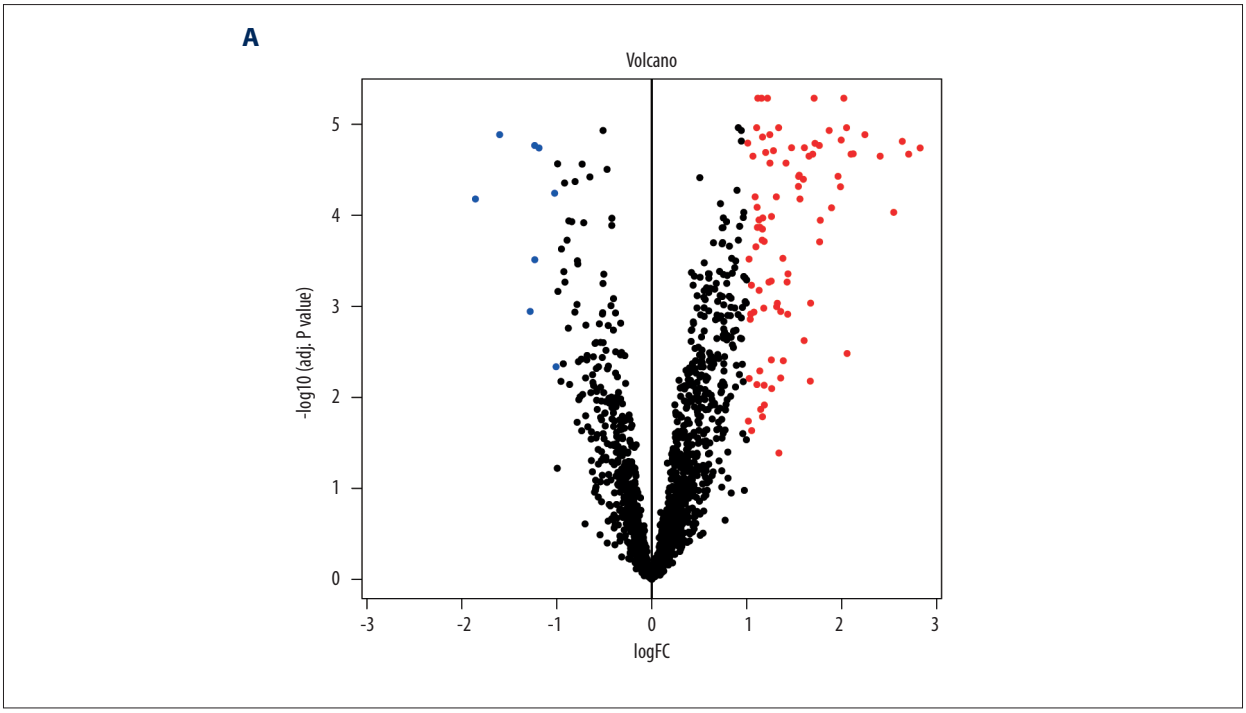
The LASSO regression algorithm screened 11 genes from 96 immune-related genes (**Figure 5A**), while the SVM-RFE algorithm screened 16 genes from 96 immune-related genes (**Figure 5B**). The same 7 genes in the results of the 2 algorithms were CASP10, FCGR3A, C1QB, XBP1, SERPINB9, CHP2, and LGR4 (**Figure 5C**).

ROC Curves of 7 Specifically Expressed Immune-Related Genes in Peri-Implantitis and Normal Tissues

ROC curve analysis is a statistical tool widely used in the medical field to predict diagnostic efficacy [28]. The results showed the 7 specific immune-related genes that were highly correlated with peri-implantitis. Among them, FCGR3A showed the highest value in peri-implantitis (area under the ROC curve [AUC]=0.964). The values of other genes were as follows: C1QB (AUC=0.961), CASP10 (AUC=0.947), SERPINB9 (AUC=0.941), LGR4 (AUC=0.938), CHP2 (AUC=0.934), and XBP1 (AUC=0.934) (**Figure 6**). The results indicated that the screened genes had strong diagnostic ability.

Immune Infiltration Analyses

The infiltration of immune cells in peri-implantitis had not been thoroughly studied previously. Here, we described the infiltration of immune cells in peri-implantitis through ssGSEA (**Figure 7A**). Results showed that activated B cells, activated B cells, activated CD8 T cells, activated dendritic cells, eosinophilia, gamma delta T cells, immature B cells, immature dendritic cells, mdscna, macrophagena, mast cells, monocytena, natural killer T cells, neutrophilia regulatory T cells, type 1 T-helper cells, T-follicular helper cells ($P<0.01$), and type 17



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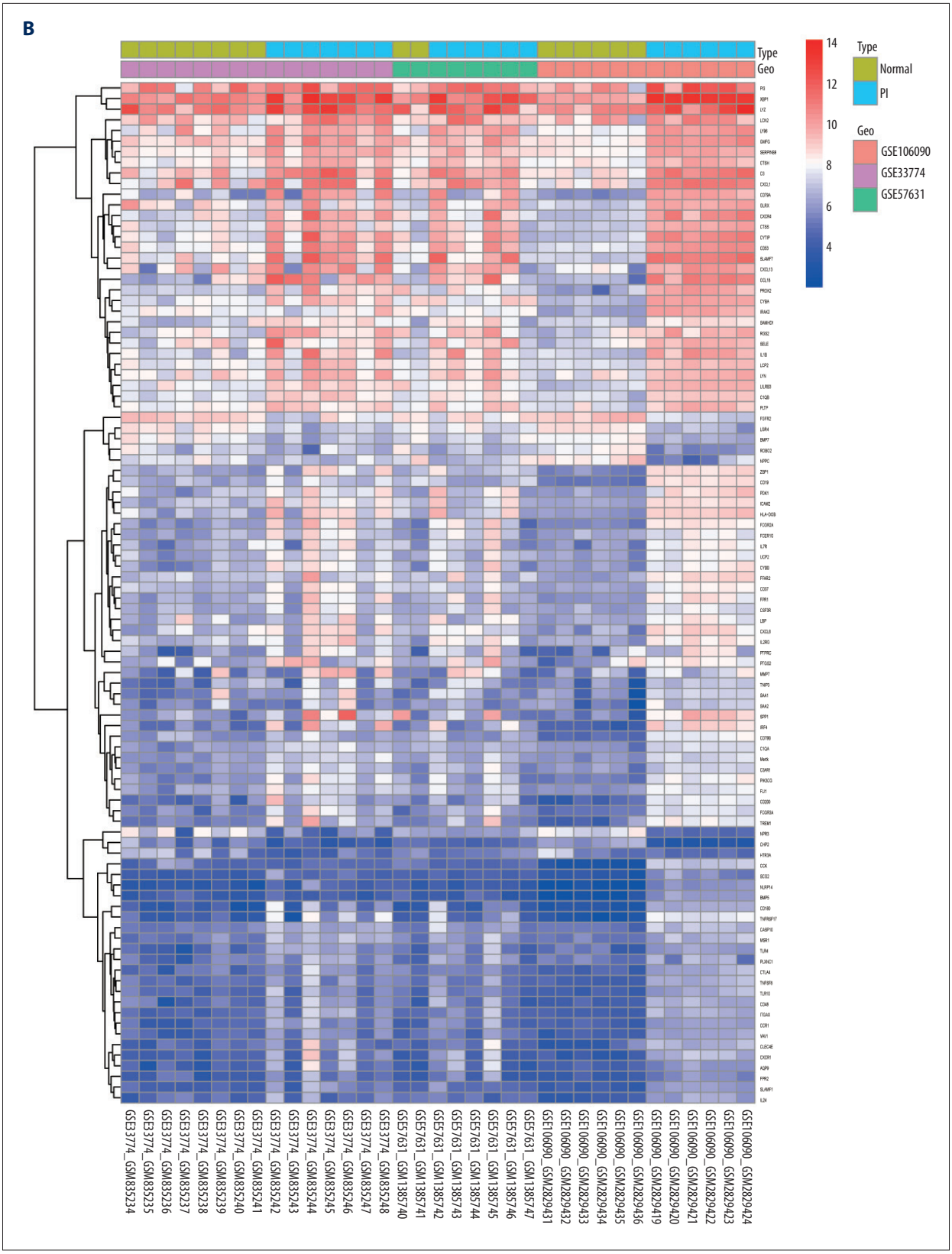

Figure 3. (**A, B**) A volcano plot and heat map showing the 96 immune-related differentially expressed genes.

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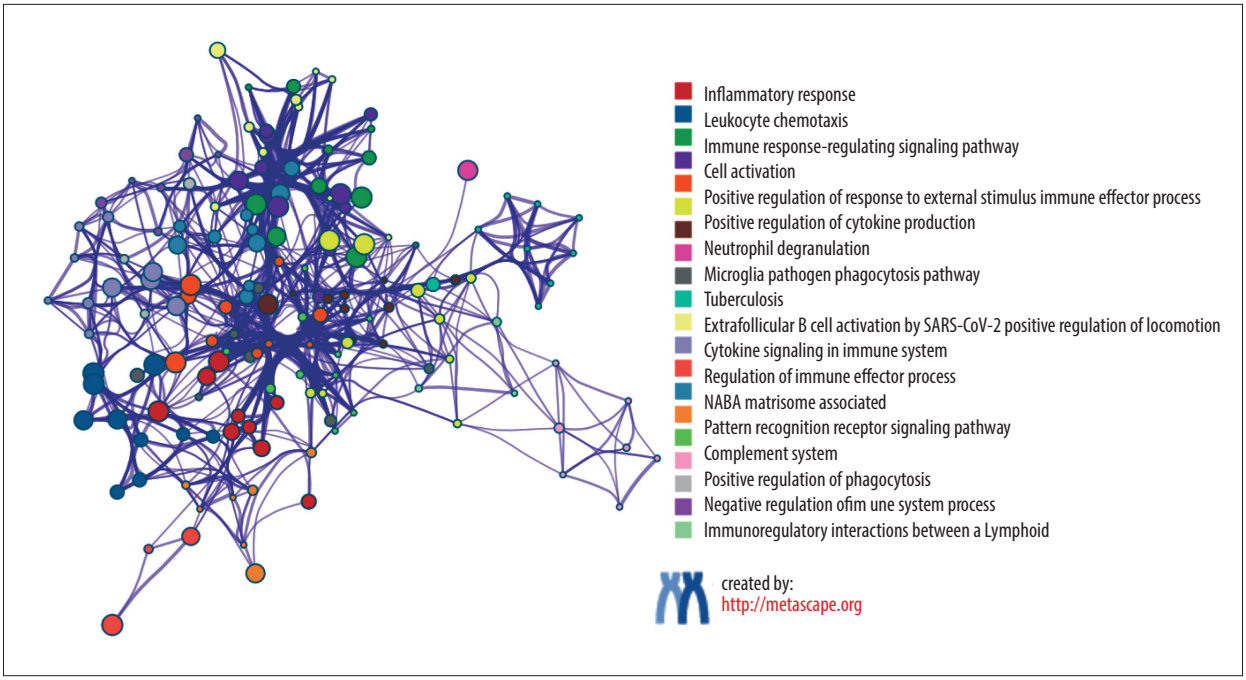

Figure 4. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of differentially expressed genes, the top 4 terms are inflammatory response, leukocyte chemotaxis, immune response-regulating signaling pathway, and cell activation.

T-helper cells ($P<0.05$) in peri-implantitis tissues were higher than that in normal tissues, and the difference was statistically significant. Also, the changes of type 2 T-helper cells, CD56 bright natural killer cells, CD56 dim natural killer cells, and plasmacytoid dendritic cells were not obvious. In addition, we analyzed the correlation between immune infiltration and key genes. Spearman correlation analysis showed that CASP10, FCGR3A, C1QB, XBP1, and SERPINB9 were positively correlated with the above immune cells, while CHP2 and LGR4 were negatively correlated (**Figure 7B**).

Validation of Hub Gene Expression

To confirm the abnormal expression of genes in peri-implantitis, clinical samples were used to verify the results of bioinformatics analysis. The qRT-PCR analysis showed that the gene expression was consistent with the results of the bioinformatics analysis (**Figure 8**).

The expressions of CASP10, FCGR3A, C1QB, XBP1, and SERPINB9 were significantly increased, while the expressions of CHP2 and LGR4 were significantly decreased. The differences were statistically significant.

Discussion

Although peri-implantitis has the same pathogenic factors (plaque) and similar pathogenesis as periodontitis, peri-implantitis and periodontitis are not the same disease, both in the bacterial composition of plaque and the speed of tissue destruction [29]. Peri-implantitis can lead to the loss of the alveolar bone supporting the implant and ultimately lead to the failure of the implant treatment, in which immune response plays a key role [30]. Exploring the immune inflammatory characteristics of peri-implant diseases is helpful for the personalized prevention and treatment of patients. This study was the first to jointly study 3 peri-implantitis datasets (including 19 peri-implantitis tissue samples and 16 normal tissue samples). A total of 398 DEGs were found, a total of 96 immune-related genes were screened out by ImmPort and InnateDB, and 7 immune-related genes closely related to peri-implantitis were screened out by LASSO and SVM-RFE in the first peri-implantitis-related study.

Periodontal pathogens are the initial factors of peri-implantitis, but the tissue damage caused by the immune response can be more serious than that caused by pathogenic bacteria. Therefore, it is important to explore the role of the immune microenvironment in the occurrence and development of peri-implantitis. The immune response plays a key role in alveolar bone resorption. Galarraga-Vinueza et al [31] found that macrophage infiltration is increased in peri-implantitis tissues, and the proinflammatory phenotype of macrophage M1 is increased in the advanced stage of peri-implantitis. The enrichment analysis of GO and KEGG in this study showed that DEGs were mainly enriched in informational response, leucocyte chemotaxis, immune response-regulating signaling pathway, and

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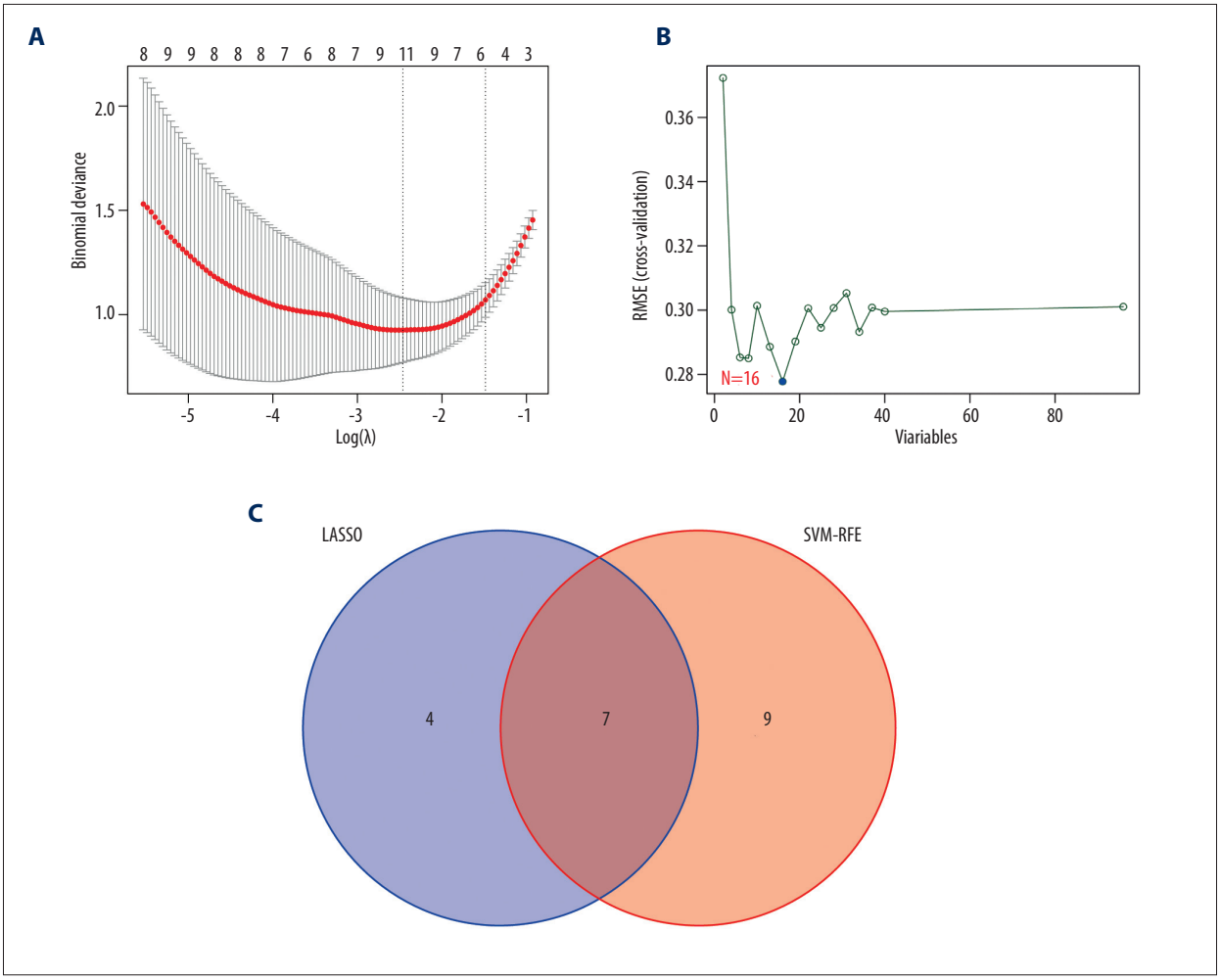

Figure 5. Screening of immune-related genes. (**A**) Least absolute shrinkage and selection operator (LASSO) logistic regression algorithm to screen differentially expressed immune-related genes. (**B**) Support vector machine-recursive feature elimination (SVM-RFE) algorithm to screen differentially expressed immune-related genes. (**C**) Venn diagram demonstrates the intersection of differentially expressed immune-related genes obtained by the 2 algorithms.

cell activation. Also, the relationship between peri-implantitis and immune response was verified again.

Because they have been limited by research methods, previous studies have only been able to investigate the expression of genes related to peri-implantitis tissue in a small dimension. In this study, we used bioinformatics methods to study the DEGs between peri-implantitis tissue and normal tissues from the perspective of overall gene expression in tissues. Seven genes related to peri-implantitis (CASP10, FCGR3A, C1QB, XBP1, SERPINB9, CHP2, and LGR4) were screened out, thus providing a direction for the exploration of peri-implantitis-related pathogenesis, molecular diagnosis, and gene therapy.

Among the above genes, FCGR3A had the highest AUC value (AUC=0.964), indicating that it was highly correlated with peri-implantitis, which is worthy of further study. Immunoglobulin G (IgG) is an important part of the immune system. Studies on immunoglobulin G are very extensive and have been used to develop a variety of new therapeutic antibodies, especially in malignant tumors and other autoimmune-related diseases [32]. FCGR is the Fc receptor of immunoglobulin G. The FCGR gene family has been proven to be closely related to many diseases characterized by bone absorption and autoimmune diseases, such as periodontitis, rheumatoid arthritis, pemphigus, systemic lupus erythematosus, and neuromyelitis optica [32-38]. Studies have shown that FcRg-mediated ITAM signaling is a key pathway in osteoclastogenesis, which is also consistent with the results of the present study [39].

Tohmonda et al [40] showed that the IRE1α/XBP1 pathway, as a key regulator, promoted osteoclastogenesis by enhancing Nfatc1 transcription, and inhibition of the IRE1α/XBP1 pathway can help to inhibit pathological bone resorption. Also, previous studies have

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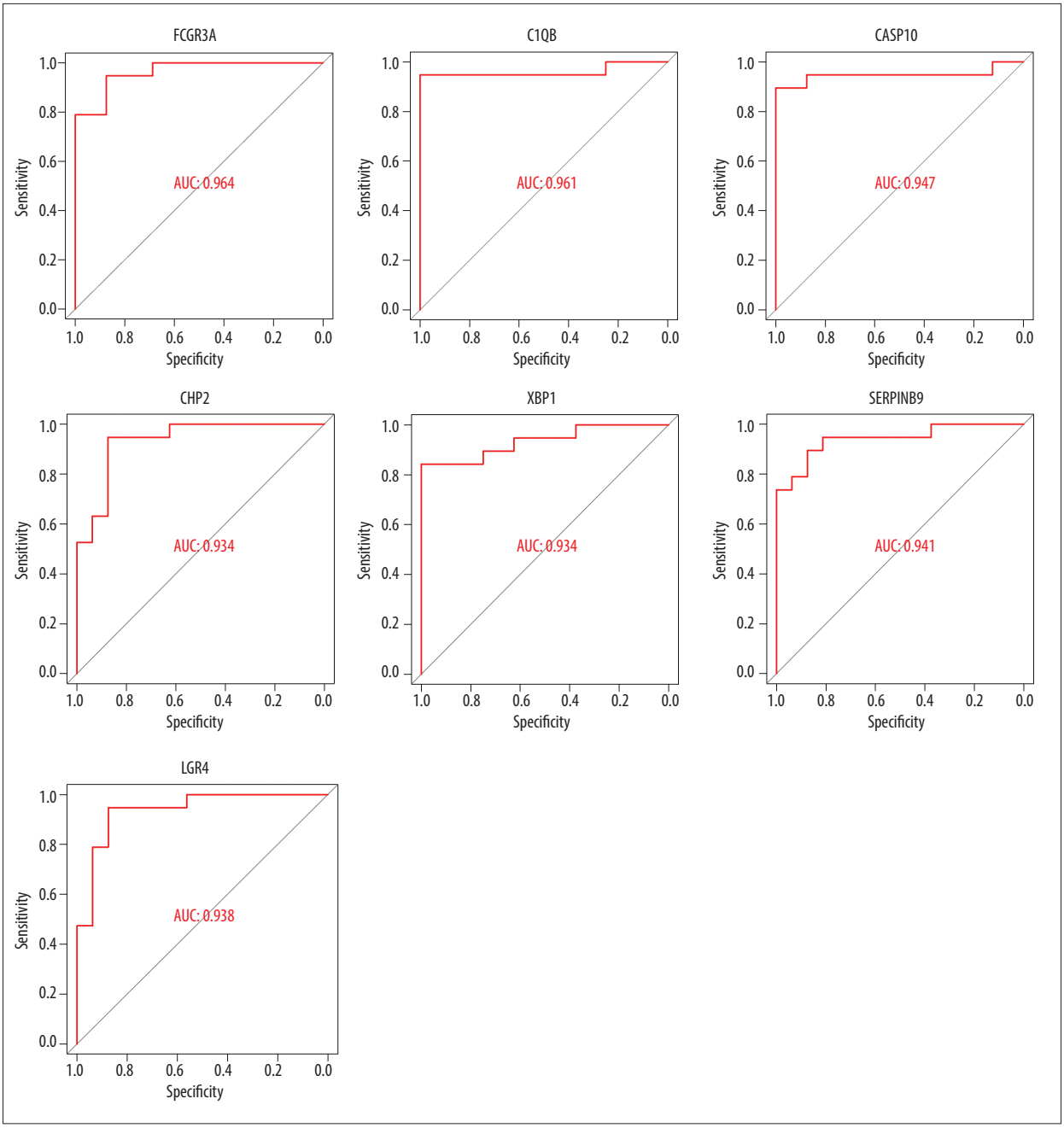

Figure 6. Receiver operating characteristic curve of the 7 specifically expressed hub genes in peri-implantitis and normal tissues.

detected the presence of C1q deposits in gingival tissues of patients with periodontitis [41]. Kuramoto et al [42] found that the expression of C1qB was enhanced in the bound epithelium and the connective tissue near the bound epithelium in the acute phase of a rabbit experimental periodontitis model. SERPINBB9, a common proinflammatory factor of IL-1β [43], can regulate the release of IL-1β in human monocytes and reduce the formation of IL-1β by inhibiting caspase-1 [44]. Decreased SERPINB9 expression in peri-implantitis may be related to increased IL-1β production. The Nf-κB signaling pathway is a crucial pathway in the process of osteoclast activation and differentiation [45]. CASP10 can promote DISC-mediated gene induction and activate the NF-κB signaling pathway [46]. All of these results are consistent with the results of the present study. CHP2 was significantly downregulated in peri-implantitis tissues, compared with normal tissues. To date, a few studies have focused on the biological function of CHP2, showing that CHP2 can active the calcineurin/nuclear factor of activated T-cell signaling pathway, which can regular bone homeostasis. This suggests that CHP2 can play a role in the process of peri-implantitis bone resorption [47,48].

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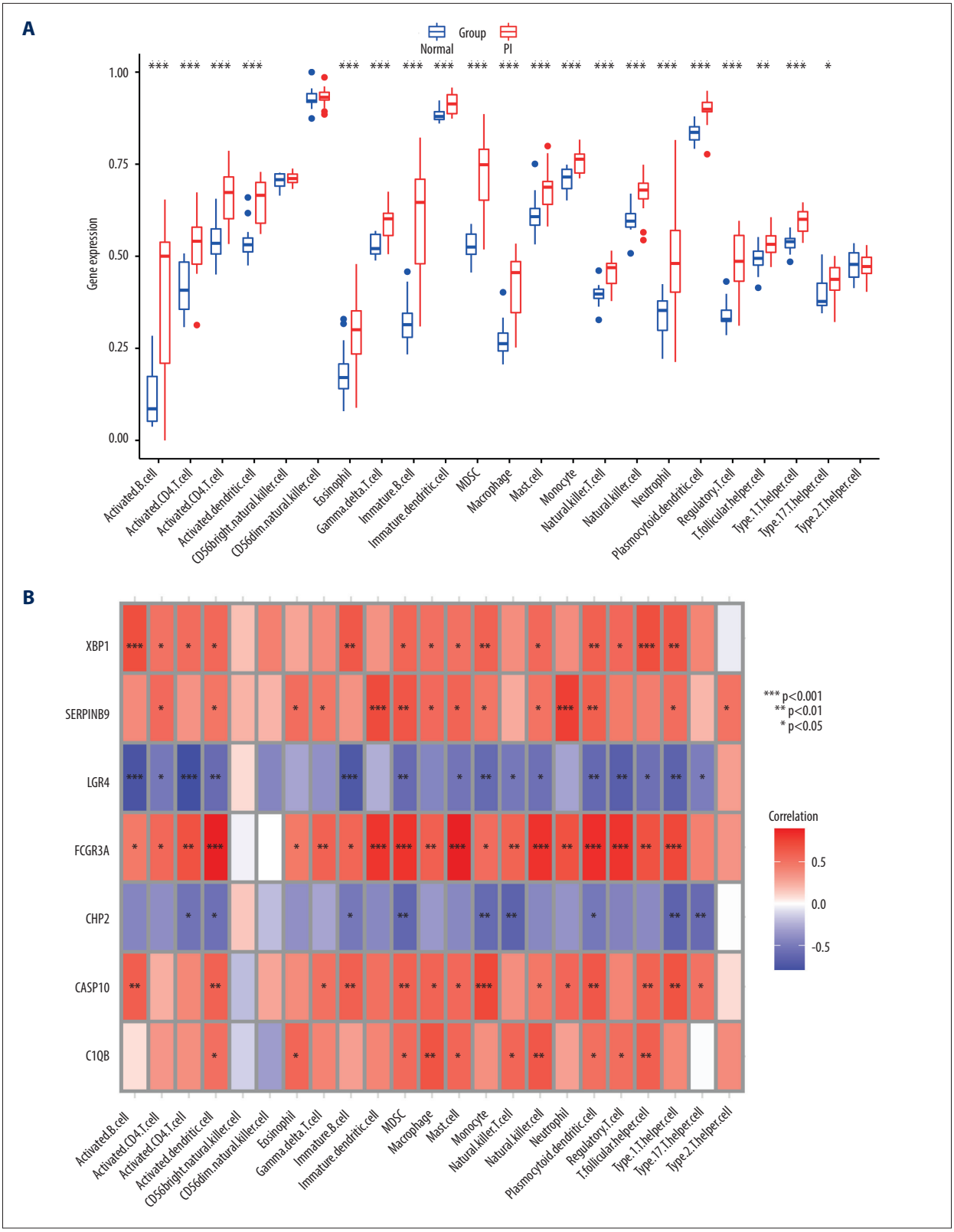

Figure 7. (**A**) Box plots of immune infiltration profiles. (**B**) Correlation between key genes and infiltrating immune cells. A positive correlation is represented by red, while a negative correlation is represented by blue. The significant differences are marked by "*".

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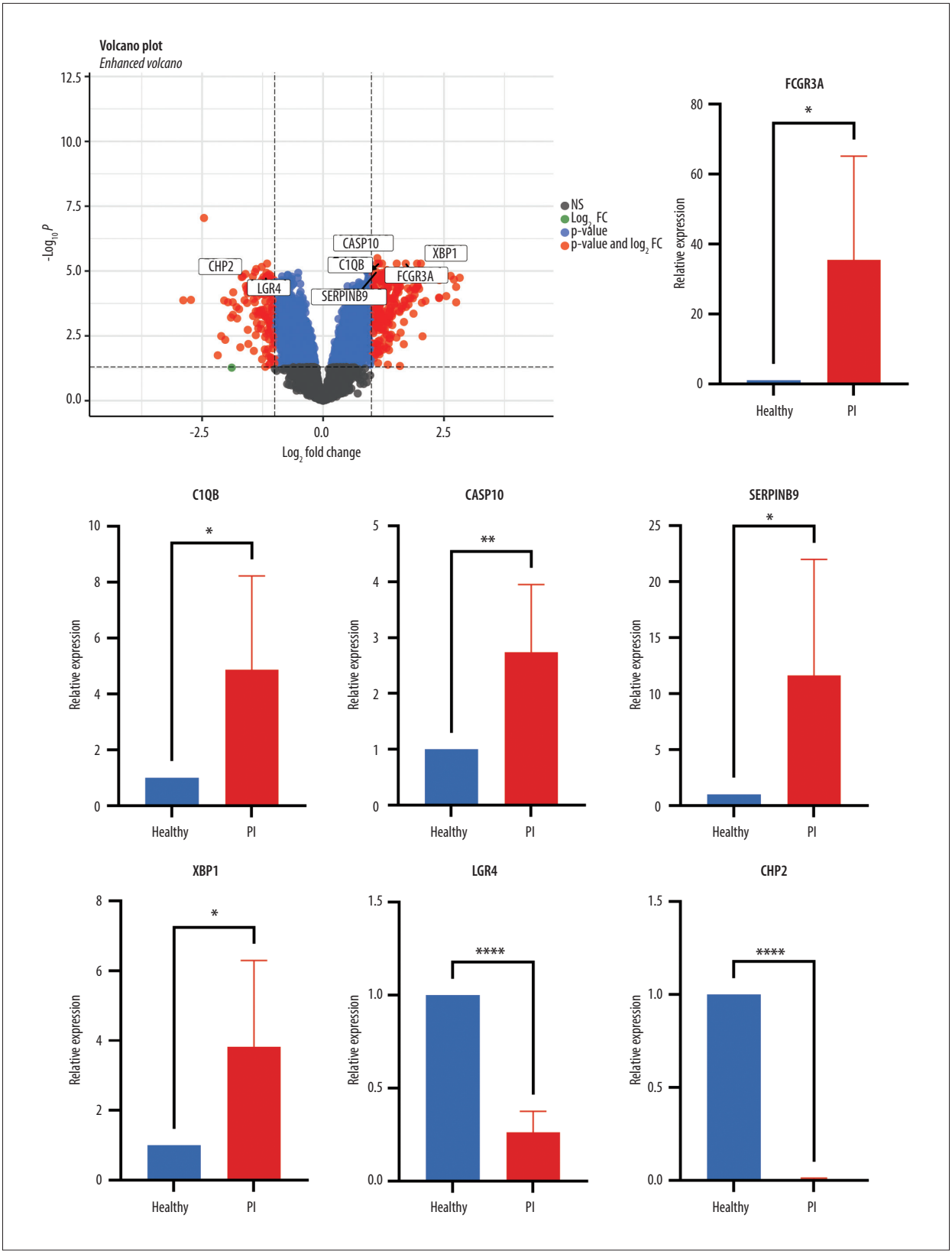

Figure 8. Expression of 7 hub genes in human gingival tissues between normal and peri-implantitis groups. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

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It is worth noting that many studies have shown that LGR4 is closely related to bone remodeling. LGR4 regulates bone formation and remodeling through the cAMP-PKA-Atf4 signaling pathway [49]. In addition, LGR4 plays an important role in osteoblast activity, and the lack of LGR4 osteoblasts impairs the aerobic glycolysis ability and leads to the reduction of new bone formation [50]. LGR4 can competitively bind RANKL with RANK and inhibit this pathway during osteoclastogenesis, and LGR4 has little effect on pre-osteoclasts, while affecting mature osteoclasts. Therefore, targeting LGR4 may reduce the related adverse effects. At the same time, RANKL combined with LGR4 can also activate the Gαq and GSK3-β signaling pathway, which can inhibit the expression of nuclear factor of activated T cells, cyclotomic, and calcineurin-dependent 1 in osteoclastogenic activity [51]. Therefore, we hypothesized that targeting LGR4 may be also a key target to reduce the peri-implantitis-induced bone loss.

The European Federation of Periodontology/American Academy of Periodontology clearly defined peri-implantitis, including bleeding/suppuration on probing, increasing probing depth and progressing crestal bone loss [52]. However, the diagnostic indicators of peri-implantitis have some limitations: bleeding on probing has large variability, the healthy tissues around the implant sometimes also have bleeding on probing, other studies have shown that probing depth is not related to the prevalence of peri-implantitis, and accurate diagnosis and timely intervention of peri-implantitis are very important [53]. Molecular diagnosis can enhance the accuracy of peri-implantitis diagnosis and contribute to personalized treatment of patients [54]. The present study will not only help to identify the genes that play a role in the immune mechanism of peri-implantitis, but also provide important targets for the early molecular diagnosis of peri-implantitis. The ROC curve is an analytical method, represented graphically, used to evaluate the performance of binary diagnostic classification methods [55]. The AUC value is the area under the ROC curve, which can accurately reflect the diagnostic performance of a certain method. An AUC value >0.7 is considered acceptable, while an AUC value >0.9 is considered to have excellent diagnostic performance [56]. According to the results of this study, the AUC values of all screened genes are greater than 0.9, and some are even greater than 0.95 (FCGR3A: AUC=0.964, c1qb: AUC=0.961). These results indicate that these genes have high value in the molecular diagnosis of peri-implantitis.

Conclusions

We found 7 genes closely related to peri-implantitis (CASP10, FCGR3A, C1QB, XBP1, SERPINB9, CHP2, and LGR4) through bioinformatics and qRT-PCR. Among them, CASP10, FCGR3A, C1QB, XBP1, and SERPINB9 were significantly increased in peri-implantitis gingival tissues, while CHP2 and LGR4 were significantly decreased. Also, peri-implantitis was closely related to inflammatory response, leukocyte chemotaxis, immune response-regulating signaling pathway, and cell activation. These results have deepened our understanding of peri-implantitis. The specific role of these genes in the development of peri-implantitis needs to be further studied.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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