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Research paper

Regulatory role of PDK1 via integrated gene analysis of mitochondria-immune response in periodontitis

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ABSTRACT

Aims: To investigate the association between mitochondrial events and immune response in periodontitis and related regulatory genes.

Main methods: Gene expression profiles in gingival tissues were retrieved from the Gene Expression Omnibus. Mitochondria-immune response-related differentially expressed genes (MIR-DEGs) between the healthy and periodontitis samples were determined. WGCNA, GO, and KEGG were used to investigate the function and the enriched pathways of MIR-DEGs. The correlation between MIR-DEGs expression and clinical probing pocket depth was analyzed. The MIR-DEGs were further identified and verified in animal samples. A periodontitis model was established in C57BL/6 mice with silk ligation. Micro-computed tomography was used to assess alveolar bone loss. Western blot, quantitative real-time polymerase chain reaction, and immunohistochemical analyses further validated the differential expression of the MIR-DEGs.

Key findings: A total of ten MIR-DEGs (CYP24A1, PRDX4, GLDC, PDK1, BCL2A1, CBR3, ARMCX3, BNIP3, IFI27, and UNG) were identified, the expression of which could effectively distinguish patients with periodontitis from the healthy controls. Enhanced immune response was detected in the periodontitis group with that in the healthy controls, especially in B cells. PDK1 was a critical MIR-DEG correlated with B cell immune response and clinical periodontal probing pocket depth. Both animal and clinical periodontal samples presented higher gene and protein expression of PDK1 than the control samples. Additionally, PDK1 colocalized with B cells in both animal and clinical periodontal tissues.

Significance: Mitochondria participate in the regulation of the immune response in periodontitis. PDK1 may be the key mitochondria-related gene regulating B-cell immune response in periodontitis.

Abbreviations: MIR-DEGs, Mitochondria-immune-related differentially expressed genes; PDK1, Pyruvate Dehydrogenase Kinase Isozyme 1; PRDX4, Peroxiredoxin-4; DEGs, Differentially expressed genes; WGCNA, Weighted correlation network analysis; GEO, Gene Expression Omnibus; ssGSEA, Single-sample gene-set enrichment analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, The receiver operating characteristic; AUC, The area under the curve; GSEA, Gene set enrichment analysis; PDL, periodontal ligament; μCT, Micro-computed tomography; CEJ, Enamel-cemental junction; ABC, Alveolar bone crest; BMD, Bone mineral density; BV/TV, Bone value/total value; qRT-PCR, Quantitative real-time polymerase chain reaction; mRNA, Messenger RNA.

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1. Introduction

Periodontitis is a prevalent inflammatory disease that affects the integrity of tooth-supporting tissues (Hajishengallis and Chavakis, 2021). Periodontitis affects approximately 743 million people worldwide (Peres et al., 2019). Periodontitis, the leading cause of tooth loss in adults, imposes significant healthcare, social, and economic burdens (Genco and Sanz, 2020). Moreover, periodontitis dramatically increases the risk of developing systemic diseases such as diabetes and cardiovascular diseases (Peres et al., 2019). However, most of the current treatments for periodontitis are ineffective (Di Stefano et al., 2022). Therefore, it is imperative to further clarify the pathological mechanisms underlying periodontitis and identify new effective therapies against periodontitis.

Accumulating evidence has confirmed the essential role of mitochondrial dysfunction in periodontitis progression (Chen et al., 2019; Sun et al., 2017). Clinical studies have revealed abnormal mitochondrial structure and function within gingival tissues and human gingival fibroblasts from patients with periodontitis compared with those of healthy individuals (Zhang et al., 2021). Studies have also revealed that the excessive production of mitochondrial reactive oxygen species, imbalanced mitochondrial dynamics, and mitochondrial DNA damage affect the periodontitis progression (Jiang et al., 2023). Furthermore, the targeted modulation of mitochondria with mitochondrial division inhibitor 1 and hydroxytyrosol has shown great potential for attenuating periodontitis (Zhang et al., 2021; Shi et al., 2021). More impressively, mitochondrial dysfunction may be a shared pathology that links periodontitis with systemic diseases, including neurodegenerative diseases (Johnson et al., 2021), obesity (de Mello et al., 2018), and diabetes mellitus (Pinti et al., 2019). However, the specific role of mitochondrial dysfunction in periodontitis remains unclear.

Compelling evidence indicates that mitochondria are at the heart of immunity (Breda et al., 2019). Mitochondrial biogenesis and dynamics regulate immune cell activation, differentiation, inflammatory responses, and survival (Faas and de Vos, 2020). T and B cells require reactive oxygen species (ROS), which are mainly produced by the mitochondria, to generate appropriate immune responses (Liu and Ho, 2018). Furthermore, mitochondrial dysfunction underlies the pathogenesis of various immune-mediated diseases, including Alzheimer's disease, cardiovascular disease, and diabetes mellitus (Prasun, 2020; Beck et al., 2019). An aberrant immune response is also considered a significant cause of periodontitis, with plasma cells, B cells, and macrophages being the central immune cells involved (Figueredo et al., 2019). Clinical and animal studies have revealed that samples from patients with gingivitis and periodontitis showed more immune cell infiltration than that of the healthy samples (Garaicoa-Pazmino et al., 2019). In vitro experiments have also confirmed that dysregulated immune cells directly reinforced inflammation, activated osteoclastic activity, and subsequently lead to tissue destruction (Kurgan and Kantarci, 2018). Furthermore, immunotherapy is effective for repairing and regenerating periodontal tissues (Yang et al., 2021). Collectively, dysregulated immune responses play a crucial role in periodontitis progression. However, the relation between mitochondria and immune response in periodontitis remains unclear.

In this study, we explored the possible role of the mitochondria in regulating the immune response in periodontitis and identified the involved regulatory genes. Our study provides novel insight into the mitochondria-immune response link in periodontitis and opens avenues for the development of therapeutic targets and drugs for periodontitis.

2. Materials and methods

2.1. Bioinformatics analysis

2.1.1. Data acquisition

Two publicly available datasets were obtained from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). The GSE16134 dataset, which included 241 periodontitis samples and 69 healthy samples, was used as the analysis and test cohort. GSE10334 was used as the validation cohort, involving 183 periodontitis and 64 healthy samples. A total of 1035 human mitochondrial genes were extracted from MitoCarta3.0 (www.broadinstitute.org/pubs/MitoCarta) (Demmer et al., 2008; Kebschull et al., 2014).

2.1.2. Analysis of differentially expressed genes

The R package "limma" was applied to explore genes with $|\log_2 FC| > 0.5$ and an adjusted *p-value* < 0.05 as differentially expressed genes (DEGs) between the healthy and periodontitis samples (Ritchie et al., 2015). The intersection of the mitochondrial genes and DEGs was identified as mitochondria-related DEGs. Single-sample gene-set enrichment analysis (ssGSEA) was performed with the R package "GSVA", which calculated mitochondria scores based on the expression of mitochondria-related DEGs (Ferreira et al., 2021).

2.1.3. Weighted correlation network analysis (WGCNA) and functional enrichment analysis

Gene expression profiles from the merged cohort and sample traits (low- and high- mitochondria score) were analyzed using WGCNA (Liu et al., 2021). A soft threshold ($\beta = 3$, scale-free $R^2 = 0.95$) was applied to guarantee the scale-free networks. The Pearson correlation coefficient between the module eigengenes and sample traits determined the most relevant module (hub module). Furthermore, Gene Ontology (GO) analysis of the DEGs was conducted using the DAVID and Metascape databases (https://metascape.org), which explored the biological process, cellular component, molecular function, and enrichment pathways related to the DEGs. Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) was constructed using KOBAS 3.0 (https://kobas.cbi.pku.edu.cn/anno_iden.php) to investigate pathways enriched with DEGs. $p < 0.05$ represented statistically significant differences (Carbon et al., 2009; Kanehisa et al., 2017).

2.1.4. Construction and validation of the classification model

The intersection of genes in the hub module and mitochondria-related DEGs was identified as mitochondrial immune response-related DEGs (MIR-DEGs). Logistics analysis was performed on the validation dataset to construct a classification model based on the MIR-DEGs (Noble, 2006). The receiver operating characteristic (ROC) curve was used to evaluate the model classification accuracy. The model was then verified using the GSE10334 dataset. A nomogram was established to predict the risk of periodontitis in patients based on the feature genes using the "rms" R package. The predictive efficacy of the nomogram was estimated using calibration curves.

2.1.5. Evaluation of infiltrating immune cells and immune-related pathway

Immune cell profiles were analyzed using the CIBERSORT algorithm (Newman et al., 2015). Differences in the proportions of immune cell subgroups between the control and periodontitis groups were quantified. The infiltrating scores of 22 types of immune cells were plotted via single-sample gene-set enrichment analysis (ssGSEA) using the R package "GSVA" with the related marker genes shown in Table S1. Marker genes of the immune-related pathways were shown in Table S2.

2.1.6. Single gene functional pathway enrichment analysis

Periodontitis samples were divided into low- and high-expression groups based on the expression of MIR-DEGs. The DEGs between the two groups were identified by $|\log FC| > 0.5$ and an adjusted *p-value* < 0.05. Subsequently, we performed gene set enrichment analysis (GSEA) with the R package "cluster Profiler" (Yu et al., 2012). All DEGs were evaluated using GO and KEGG enrichment analysis.

2.2. Collection of clinical tissue specimens

The Ethics Committee of the Wenzhou Medical University approved the experimental procedures (No. WYKQ2022019). Gingival tissues were obtained from the Affiliated Stomatology Hospital of Wenzhou Medical University between January 2022 and February 2024. 12 women and 14 men aged 23–78 years were enrolled. Healthy individuals presented probing pocket depth of < 4 mm, no bleeding on probing, and no signs of periodontal tissue damage. Patients with periodontitis conformed to the periodontitis classification, with more than 30 % periodontal bone loss among all the teeth, and at least two teeth with ≥ 6 mm deep pockets, attachment loss ≥ 5 mm, and radiographic bone loss exceeding 1/2 of the root length (Bao et al., 2022). Exclusion criteria included patients who were pregnant or nursing, cigarette smokers had received antibiotic or periodontal therapy in the previous three months, had systemic conditions affecting the periodontal progression, or had any condition requiring antibiotic coverage during the periodontal therapy (Lang et al., 2015). Sociodemographic variables included age and sex. Smoking status was used as a behavioral variable. Periodontal probing pocket depth was included as an oral clinical variable (Gürsoy et al., 2022).

2.3. Animal allocation and establishment of periodontitis model

Twelve male C57BL/6 mice (6–8 weeks) were acclimated for one week and separated into the control and periodontitis groups. We ligated the bilateral upper second molars with a sterile 5–0 black braided nylon thread (Surgilon; USS/DG, Norwalk, CT, USA) and maintained them in a submarginal position for two weeks. All the mice were euthanized and their maxillae were extracted for further examination (Hao et al., 2015). All the animal-related experiments were approved by the Animal Ethics Committee of the Wenzhou Medical University (WYDW2019-0665).

2.4. Animal histological analysis and immunological staining

The right maxillae were excised, fixed with paraformaldehyde, and scanned using micro-computed tomography (μCT) to evaluate bone resorption. The area between the cemental-enamel junction (CEJ) and the alveolar bone crest (ABC), bone mineral density (BMD), and bone value/total value (BV/TV) of the alveolar bone around the second molar were measured as previously described (Chen et al., 2021).

The left maxillae were fixed, decalcified, and embedded in paraffin to investigate the inflammatory cell infiltration (Li et al., 2018). Approximately 4-μm-thick serial sections were made from paraffin-embedded tissue block and stained with hematoxylin-eosin. Furthermore, the protein level of Pyruvate Dehydrogenase Kinase Isozyme 1 (PDK1) in mice were investigated by immunohistochemical staining. The slides were then incubated with an anti-PDK1 primary antibody (1:200, Abcam, USA). Representative regions for each site were the alveolar bone crest, gingival connective tissue, and sulcular epithelium. The brown-positive cells were observed under a microscope and evaluated using the Image J software.

2.5. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

For messenger RNA (mRNA) expression analysis, gingivae were harvested from the gingival tissues of mice and clinical patients, and snap-frozen in liquid nitrogen. RNA was extracted with RNA Extraction Kit (Takara, Japan). Reverse transcription was performed using the Prime Script RT Master Mix Kit (Takara, Japan). Messenger RNA levels were determined using the TB Green Premix Ex Taq Kit (Takara, Japan). Primers used are listed in Table S3. The relative gene expression was calculated by the comparative $2^{-\Delta\Delta Ct}$ method and normalized to that of actin.

2.6. Western blot analysis and immunofluorescent staining

Total protein was extracted from clinical samples using a Total Protein Extraction Kit. The protein concentration in the supernatant was measured with a BCA Protein Assay Kit (Cat#P0012S, Beyotime). During the electrophoresis, we loaded 60–80 μg of proteins along with molecular weight markers in each slot in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The gel was run for 5 min at 50 V. Then we set the voltage to 100–150 V to complete the run within approximately 1 h. The proteins were transferred onto polyvinylidene fluoride membranes after electrophoresis. The membranes were further blocked by non-fat dry milk and incubated with anti-PDK1 (1:1000) and anti-β-actin (1:8000) antibodies overnight. The membranes were then washed and incubated with secondary antibodies (Invitrogen, USA). Immunoreactive bands were visualized using an ECL chemiluminescence solution and a Chemiluminescence imaging system. Image J software was used to detect the immunoreactive band relative to the optical density.

Gingival cryosections from humans and mice were fixed with paraformaldehyde and then permeabilized with 0.2 % Triton X-100 in PBS. After blocking with 2 % donkey serum, the slides were immunostained with primary antibodies against PDK1 (1:200, Abcam, Cambridge, USA) and CD20 (ab-178945; Abcam, Cambridge, UK), followed by staining with an FITC/TRITC-conjugated secondary antibody. Finally, the slides were double-stained with DAPI to visualize the nuclei under the Nikon Eclipse 80i Epi-fluorescence microscope (DS-Ri1, Nikon, Shanghai, China).

2.7. Statistical analysis

All the statistical analyses were performed using R packages (version 4.2.1). The data were analyzed with GraphPad Pro Prism 8 (GraphPad Software, United States), and presented as means ± SEM. Student's *t*-test was used for comparisons between the two groups. The Pearson correlation coefficient test was used to assess the rank correlations among different variables. Significance was represented as, $*p < 0.05$, $**p < 0.001$, and $***p < 0.0001$. Linear regression was used to investigate the correlation between the MIR-DEGs level and clinical probing pocket depth.

3. Results

3.1. Identification of mitochondria-related DEGs in periodontitis and related biological significance

Eighteen mitochondria-related DEGs were identified, with twelve upregulated and six downregulated in the periodontitis samples compared with those in healthy samples (Fig. 1A). The mitochondria score, calculated based on the expression of mitochondria-related DEGs, showed a substantial difference between the periodontitis and healthy groups (Fig. 1B). WGCNA analysis based on 1035 mitochondrial genes further identified the genes primarily involved in mitochondria regulation. The turquoise module and included hub genes showed the strongest positive correlation with a high mitochondria score of 0.67 (Fig. 1C-D). GO enrichment analysis further revealed that genes in the turquoise module were significantly enriched in various immune-related pathways, including regulation and activation of the immune response and leukocyte migration (Fig. 1E). Consistent with the GO analysis, KEGG analysis revealed that genes in the turquoise module were enriched in immune-related pathways including cytokine-cytokine receptor interactions and chemokine signaling pathways (Fig. 1F).

3.2. Construction and validation of the classification model for periodontitis

Ten MIR-DEGs, including CYP24A1, PRDX4, GLDC, PDK1, BCL2A1,

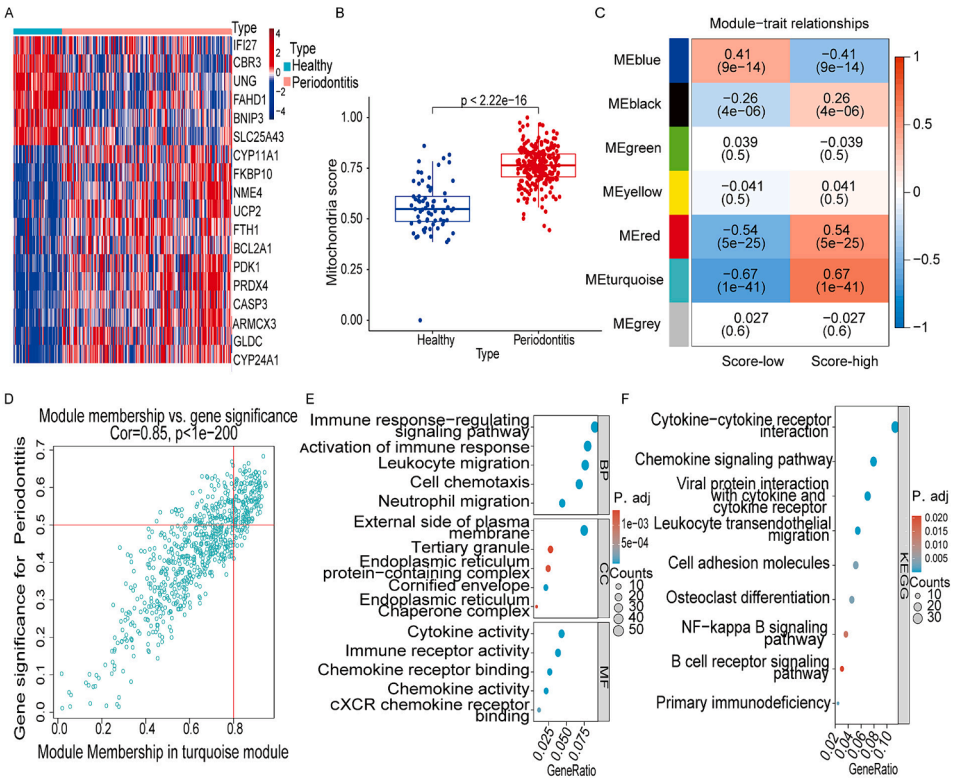

Fig. 1. Identification of mitochondrial-related differentially expressed genes (DEGs) and functional enrichment analysis. (A) Heatmap exhibited eighteen mitochondria-related DEGs between the healthy and periodontitis groups. The blue boxes indicated downregulated mitochondria-related DEGs, and the red blocks indicated upregulated mitochondria-related DEGs. **(B)** The mitochondria score was significantly different between the periodontitis and healthy groups. **(C)** Heatmap of the correlation between mitochondria score and gene modules showed that the turquoise module had the strongest correlation with a high mitochondria score. **(D)** Scatterplots of score significance versus module membership in the turquoise module. **(E)** A bar plot of the top five enriched gene ontology (GO) terms in the turquoise module for each category. BP, biological process; CC, cellular components; MF, molecular functions. **(F)** Bubble plot of the significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the turquoise module. $p < 0.05$ represented statistically significant differences.

CBR3, ARMCX3, BNIP3, IFI27, and UNG, were identified by overlapping the turquoise module genes and mitochondria-related DEGs (Fig. 2A). The expression levels of the ten MIR-DEGs in the healthy and periodontitis samples were determined (Fig. 2B). The expression levels of the ten MIR-DEGs were also verified in GSE10334 (Fig. S1). The diagnostic value of the ten MIR-DEGs for periodontitis was determined. The AUC of the ten MIR-DEGs were all over 0.7 (Fig. 2C-D). The nomogram plots were established to predict the risk of periodontitis (Fig. 2E). Each gene corresponded to a scoring criterion. A classification model was constructed based on the identified MIR-DEGs. The overall AUC of this model was 0.948, indicating that the ten MIR-DEGs had a high diagnostic value (Fig. 2F). The calibration curve of the nomogram further confirmed the good predictive performance of the model (Fig. 2G).

Additionally, we acquired GSE10334 as the validation cohort to explore MIR-DEGs. The results also showed that ten MIR-DEGs from GSE16134 were differentially expressed between healthy and periodontitis samples (Fig. S1A). The AUC of the MIR-DEGs were all larger than 0.7 (Fig. S1B–C). We further investigated the classification model based on MIR-DEGs identified from the GSE10334 database. The overall AUC was 0.926, indicating that the classification model could effectively distinguish patients with periodontitis from healthy controls (Fig. S1D–E).

3.3. Correlation between MIR-DEGs and immune response

We investigated the differential immune cell infiltrations between the healthy and periodontitis tissues from patients with periodontitis, along with the correlation between MIR-DEGs and the immune response in GSE16134. The results showed that except for Type 2 T helper cells and CD56dim natural killer cells, the infiltration of all other immune cells was significantly higher in periodontitis tissues, with activated and immature B cells exhibiting the most significant difference (activated B cell: p value = 2.5e-24; immature B cell: p value = 4.8e-21) (Fig. 3A). We also explored the immune-related pathways involved in periodontitis and their relation with MIR-DEGs. Except for the Type I IFN response, most of the immune-related pathways (11/12) showed a significant difference between the healthy and periodontitis samples indicating the involvement of the immune response in periodontitis progression (Fig. 3B). Furthermore, PRDX4, PDK1, ARMCX3, BCL2A1, GLDC, and CYP24A1 were positively linked to immune cells, whereas UNG, CBR3, BNIP3, and IFI27 showed negative correlations with immune cells. PRDX4 and PDK1 showed the highest correlation with activated and immature B cells (Fig. 3C). Correlation analysis revealed that BCL2A1, IFI27, PRDX4, PDK1, BCL2A1, ARMCX3, and GLDC were positively correlated with immune-related pathways, whereas UNG, CBR3, and BNIP3 were negatively correlated. BCL2A1 showed the highest correlation with CCR, IFI27 showed the highest correlation with para-inflammation, PRDX4 and PDK1 showed the highest correlation with T-cell co-stimulation (Fig. 3D). Similar to GSE10334, we explored differential immune cell infiltration between periodontitis samples from healthy individuals and patients with periodontitis. The correlation between MIR-DEGs and immune responses was also investigated. Except for Type 2 T helper cells, infiltration of all other immune cells was significantly higher in the periodontitis tissues, with B cells exhibiting the most significant difference (Fig. 3E). We also explored the immune-related pathways involved in periodontitis and their relation with MIR-DEGs. Most of the immune-related pathways (10/12) showed significant differences between healthy and periodontitis samples, indicating the

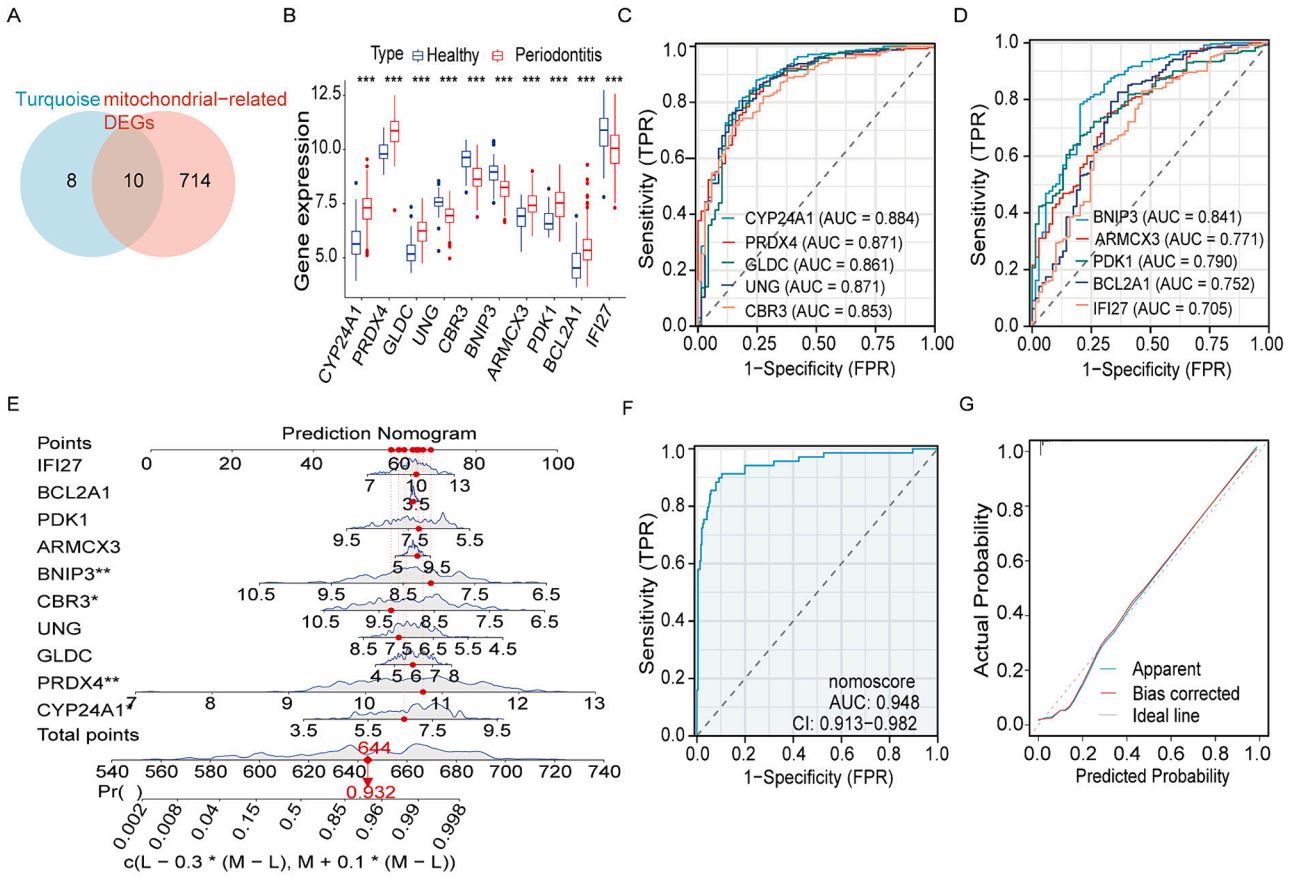

Fig. 2. Construction and validation of the classification model. (A) Intersection of the genes in the turquoise module and mitochondrial-related differentially expressed genes (MIR-DEGs). **(B)** The expression of ten MIR-DEGs showed significant differences between the healthy and periodontitis groups. **(C and D)** Receiver operating characteristic (ROC) curves showed that the classification models based on MIR-DEGs have an adequate capacity to distinguish periodontitis samples from healthy controls. **(E)** A nomogram model combined with based on ten MIR-DEGs was constructed to predict the risk of periodontitis patients. **(F)** ROC curves analysis of GSE16134 based on the diagnostic model including 10 MIR-DEGs. **(G)** Calibration curve of the nomogram explored the predictive performance of the model. $*p < 0.05$, $**p < 0.001$, and $***p < 0.0001$; ns, non-significant.

involvement of the immune response in periodontitis progression (Fig. 3F). Further correlation analysis revealed a link between MIR-DEGs and immune cell infiltration. PRDX4, PDK1, ARMCX3, BCL2A1, GLDC, and CYP24A1 were positively correlated with immune cell infiltration, whereas UNG, CBR3, BNIP3, and IFI27 were negatively correlated with immune cells; PRDX4 and PDK1 showed the highest correlation with activated and immature B cells (Fig. 3G). Correlation analysis further revealed that PRDX4, PDK1, IFI27, BCL2A1, ARMCX3, CYP24A1, and GLDC were positively related to immune-related pathways, whereas UNG, CBR3, and BNIP3 showed a negative correlation; BCL2A1 showed the highest correlation with CCR, IFI27 showed the highest correlation with Type-I-IFN-response, and PRDX4 and PDK1 showed the highest correlation with T cell co-stimulation (Fig. 3H). The specific correlation coefficients for immune cell infiltration and immune response-related pathways are shown in Fig. S2.

3.4. Exploration of functional gene modules related to MIR-DEGs

We applied WGCNA to explore the correlation patterns of DEGs in the merged cohort. The functional modules related to MIR-DEGs in periodontitis were also identified. The DEGs in the merged cohort were divided into modules by cluster analysis. After eliminating the outlier samples, six modules were identified. Ten MIR-DEGs were mainly distributed in the blue, brown, and green modules (Fig. 4A–B). Among these modules, the brown module showed the strongest relation with the MIR-DEGs (Fig. 4C). Furthermore, PRDX4 (correlation coefficient = 0.91) and PDK1 (correlation coefficient = 0.80) were closely linked to the brown module (Fig. 4D–E).

GO and KEGG analyses were conducted to clarify the function of the brown module. The GO results demonstrated that the brown module was involved in immune-related biological processes, including leukocyte migration, immune response-regulating signaling pathways, activation of the immune response, immune response-activating signal transduction, immune response-activating cell surface receptor signaling pathways, immune response-regulating cell surface receptor signaling pathways, and B cell activation (Fig. 4F). KEGG analysis further revealed that the pathways enriched in the brown module included protein processing in the endoplasmic reticulum, leukocyte *trans*-endothelial migration, leishmaniasis, chemokine signaling pathway, fc gamma R-mediated phagocytosis, osteoclast differentiation, and cytokine-cytokine receptor interactions (Fig. 4G). Considering their correlation with the brown module, PRDX4 and PDK1 may regulate mitochondrial-immune responses in periodontitis.

3.5. Analysis of the regulatory pathway related with PRDX4 and PDK1

We divided periodontitis samples into low- and high-expression groups based on the PRDX4 expression levels. We identified genes with $|\log FC| > 0.5$ and an adjusted *p-value* < 0.05 as DEGs between the low- and high-expression groups (Fig. 5A). For PRDX4, the low-expression group was predominantly enriched in olfactory transduction, linoleic acid metabolism, and drug metabolism cytochrome P450 (Fig. 5B). The high-expression group correlated with primary immunodeficiency, B cell receptor signaling pathway cells, and

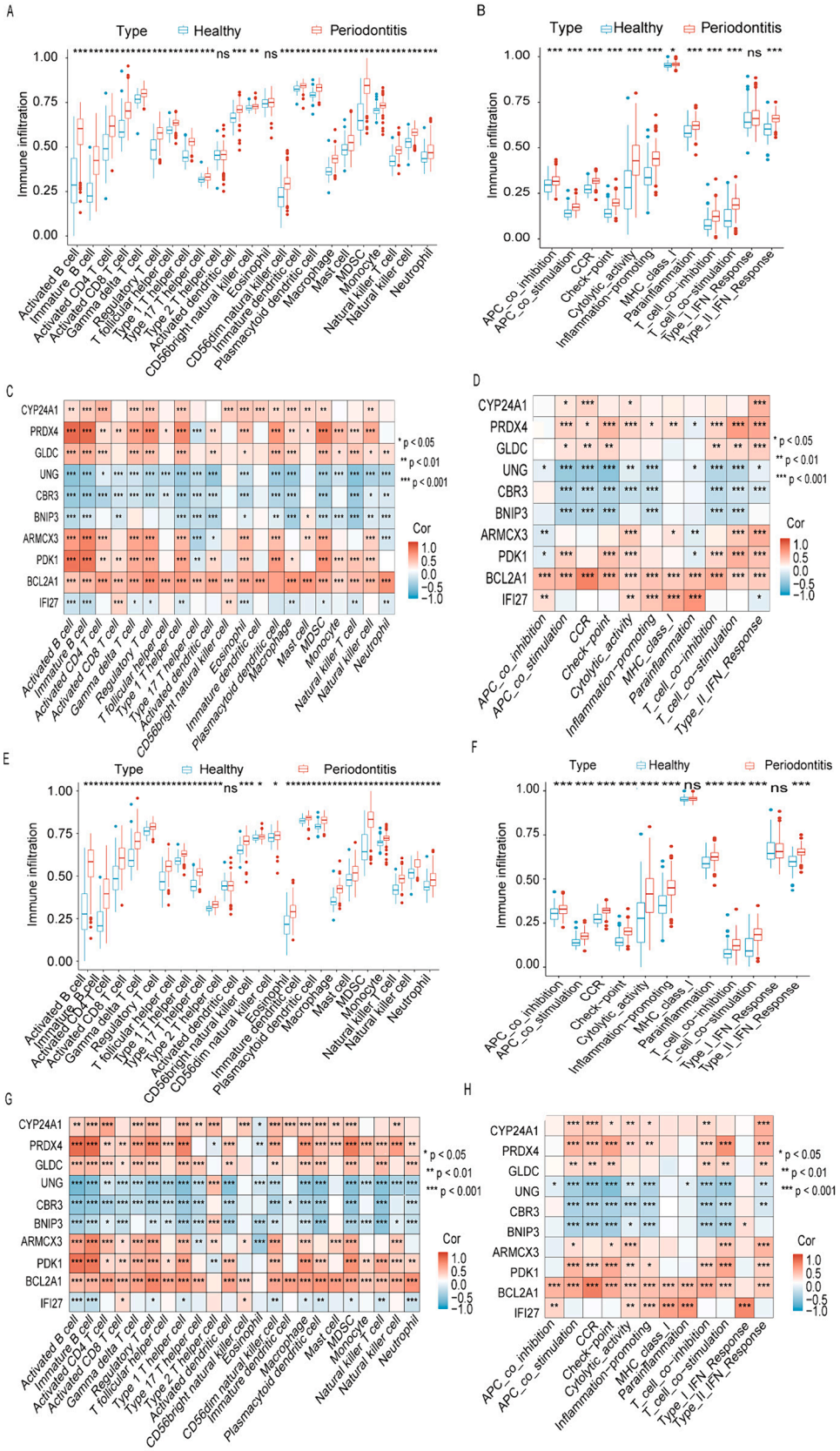

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Fig. 3. Relation between mitochondria-related differentially expressed genes (MIR-DEGs) and immune response in periodontitis. (A) Differences in infiltrating immune cell subtypes in the healthy and periodontal samples from patients with periodontitis in GSE16134. **(B)** Heatmap of *p-values* between MIR-DEGs and immune cell infiltration in GSE16134. **(C)** Correlation between MIR-DEGs and immune cell infiltration in GSE16134. **(D)** Correlation between MIR-DEGs and immune-related pathway in GSE16134. **(E)** Differences in infiltrating immune cell subtypes in the healthy and periodontal samples from patients with periodontitis in GSE10334. **(F)** Heatmap of *p*-values between MIR-DEGs and immune cells in GSE10334. **(G)** Correlation between MIR-DEGs and immune cell infiltration in GSE10334. **(H)** Correlation between MIR-DEGs and immune-related pathway infiltration in GSE10334. $*p < 0.05$, $**p < 0.001$, and $***p < 0.0001$; ns, non-significant.

adhesion molecules (Fig. 5C). GO and KEGG analysis revealed that the DEGs based on the PRDX4 level were enriched in immune response, B cell activation and B cell receptor signaling pathway, immune response-regulating signaling pathway, endoplasmic reticulum, and leukocyte transendothelial migration (Fig. 5D-F). For PDK1, periodontitis samples were also divided into low- and high-expression groups (Fig. 6A). The low-expression group was predominantly enriched in linoleic acid, butanoate, arachidonic acid metabolism, and arachidonic acid metabolism (Fig. 6B). The high-expression group was correlated with B cell receptor signaling pathway cells, primary immunodeficiency, and the intestinal immune network for IgA production (Fig. 6C). The DEGs based on the PDK1 expression were enriched in the immune response-regulating signaling pathway, B cell activation, B cell receptor signaling pathway, endoplasmic reticulum protein-containing complex, and leukocyte transendothelial migration (Fig. 6D–F). These results indicated a relationship between immune response and MIR-DEGs including PRDX4 and PDK1.

3.6. Clinical verification of MIR-DEGs and multiple regression analysis

Patients with periodontitis and the healthy individuals were enrolled to explore the expression levels of MIR-DEGs. The periodontal samples presented higher gene levels of PRDX4, PDK1, IFI27, and CYP24A1 than those of the healthy samples (Fig. 7A). Other genes showed no significant difference between the healthy and periodontitis groups. Additionally, Fig. 7A showed the differential expressions of PRDX4, PDK1, CYP24A1, GLDC, CBR3, UNG, and BNIP3 (7 out of 10 genes), were consistent with the bioinformatics analysis results shown in Fig. 1. The linear analysis further indicated a correlation between the clinical probing pocket depth and differential expression of PRDX4, PDK1, and CYP24A1, with R^2 values more than 0.7 (Fig. 7B).

3.7. Verification of MIR-DEGs in periodontal animal model

We validated the expression of MIR-DEGs in the periodontal animal model. Compared with that of mice in the control group, mice with periodontitis presented significantly aggravated alveolar bone loss, as reflected by the larger bone loss area, and reduced BMD and BV/TV of the alveolar bone around the ligated tooth (Fig. 8A–B). We detected more inflammatory cell infiltration in the periodontitis group than in the control group (Fig. 8C). Real time-polymerase chain reaction showed that the gene expressions of most MIR-DEGs (six out of ten) including PDK1, ARMCX3, BCL2A1, CBR3, UNG, and BNIP3 in mice periodontal tissues was consistent with the bioinformatic results shown in Fig. 1A (Fig. 8D). More interestingly, the increased gene expression of PDK1 in the mice periodontal tissues were aligned with both bioinformatic analysis and the clinical results ($p < 0.05$). Therefore, PDK1, rather than PRDX4, was selected for further analysis.

Compared with that in the control group, the protein level of PDK1 increased significantly both in mice with periodontitis and in the clinical gingival tissues of periodontal patients (Fig. 8E–F) ($p < 0.05$). Furthermore, the immunofluorescence staining confirmed the colocalization of PDK1 with B cells, both in animal and clinical periodontal tissues, revealing the possible regulatory role of PDK1 in B cell activity (Fig. 8G–H).

4. Discussion

Periodontitis is one of the most prevalent inflammatory diseases and the leading cause of tooth loss. Periodontitis correlates with an enhanced risk and severity of multiple systemic diseases (Bui et al., 2019), thus it is a significant health concern. Mitochondrial dysfunction plays a critical role in periodontitis progression. Mitochondria-targeting therapy is a promising approach for the treatment of periodontitis (Castejón-Vega et al., 2021). Furthermore, dysregulated host immune response is a major cause of periodontitis (Pan et al., 2019). Accumulating evidence has confirmed mitochondria as essential immune system regulators (Banoth and Cassel, 2018; Tiwari-Heckler et al., 2022). We aimed to elucidate the possible role of mitochondria in the modulation of the immune response to provide new mechanistic insight and potential drugs for the treatment of periodontitis.

We identified mitochondria-related DEGs and detected a significant difference in mitochondrial scores between the healthy and periodontitis samples, indicating the essential role of mitochondria in periodontitis development. We performed WGCNA analysis of the crucial mitochondria-related gene modules involved in periodontitis. The turquoise module was the most significant module for the mitochondrial score. Noticeably, GO and KEGG analysis confirmed that genes in the turquoise module mainly enriched in regulating the immune effector process and humoral immune response. These results provided strong bioinformatics evidence for mitochondria-related DEGs that regulate the immune response. We further investigated the relationship between mitochondria and immune response in periodontitis. Ten key mitochondria-immune response related DEGs (MIR-DEGs) were identified, most of which have not yet been studied in periodontitis. PRDX4, PDK1, and GLDC among the MIR-DEGs have been reported to modulate both mitochondria events and immune response in various diseases (De Rosa et al., 2021; Tiedemann et al., 2019; Yamada and Guo, 2018; Xie et al., 2022) and have great potential practical value. We further established a classification model based on the identified MIR-DEGs. The test cohort confirmed that the classification model could effectively discriminate patients with periodontitis from the healthy individuals in terms of mitochondrial events. These results provided compelling evidence that mitochondria played an important role in periodontitis development.

Regarding the link between mitochondrial function and immune response, we explored the relationship between MIR-DEGs and immune response. The results revealed that except for Type 2 T helper cells and CD56dim natural killer cells, the infiltrations of most immune cells were significantly enhanced in periodontitis tissues, with activated and immature B cells presenting the most significant difference. Accumulating evidence has confirmed that B cells, as a critical immune cell, play a critical role in periodontal progression (Oliver-Bell et al., 2015; Zeng et al., 2021; Hetta et al., 2020). Studies have further confirmed the dual role of B cells in regulating periodontitis progression. On the one hand, B cells play a protective role by facilitating bacterial clearance and the halting of periodontitis progression (Zeng et al., 2021). On the other hand, B cells enhanced periodontal alveolar bone destruction by secreting osteoclastogenic cytokines (Hetta et al., 2020). A literature review further suggested that B cell-targeting therapy is a new treatment for severe periodontitis (Han et al., 2022). However, the role of B cells in periodontal progression remains elusive. Our study revealed that MIR-DEGs, especially PRDX4 and PDK1, significantly correlated with B cell infiltration, indicating their potential roles in modulating B cells in

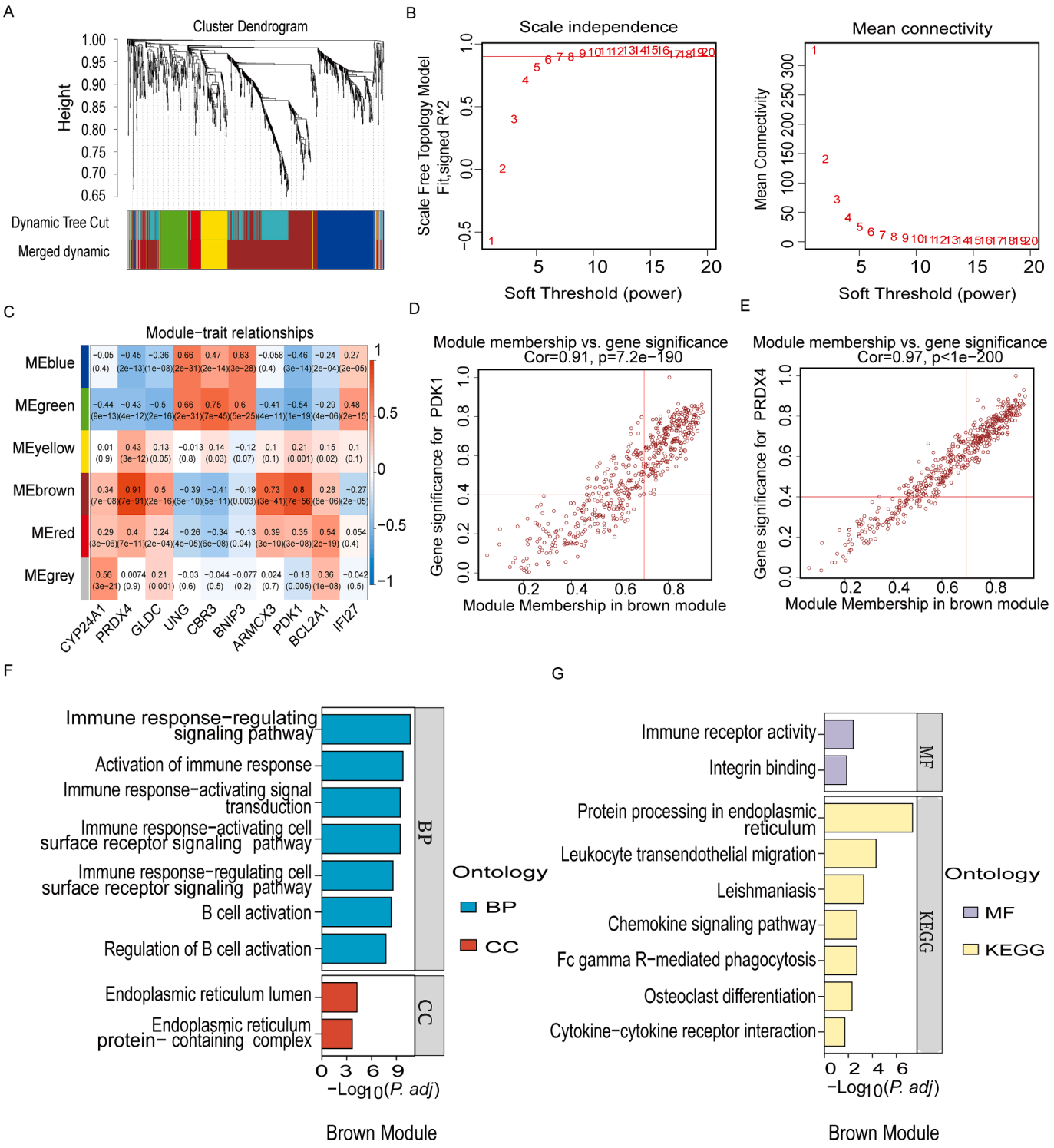

Fig. 4. Exploration of functional module linked to key mitochondrial-related genes. (A) Gene dendrogram obtained by average linkage hierarchical clustering. The color row underneath the dendrogram showed the module assignment determined by the Dynamic Tree Cut, in which 6 modules were identified. **(B)** Analysis of the scale independence and the mean connectivity for soft-thresholding powers. **(C)** Heatmap showed the six functional modules related to MIR-DEGs, including PRDX4 and PDK1. **(D)** A scatterplot of gene significance (GS) for PDK1 versus module membership in the brown module. implying that hub genes of the brown module also tend to be highly correlated with PDK1. **(E)** A scatterplot of gene significance (GS) for PRDX4 versus module membership in the brown module. implying that hub genes of the brown module also tend to be highly correlated with PRDX4. **(F and G)** Gene ontology (GO) and Kyoto Encyclopedia of Gene and Genomes (KEGG) analysis of the brown module indicated that the genes in these modules were involved in immune-related biological processes and pathways.

periodontitis. Studies have confirmed that PRDX4 may confer survival advantages to B cells (Han et al., 2019). Additionally, PDK1 is indispensable for regulating B cell survival and function (Zou et al., 2022). However, their roles in regulating B cell-mediated immunity during periodontitis require further exploration. Moreover, we found that the immune-related pathways involved in periodontitis correlated with the MIR-DEGs such as BCL2A1, CYP24A1, and ARMCX3. Therefore, the pivotal role of mitochondria in modulating the B cell-mediated

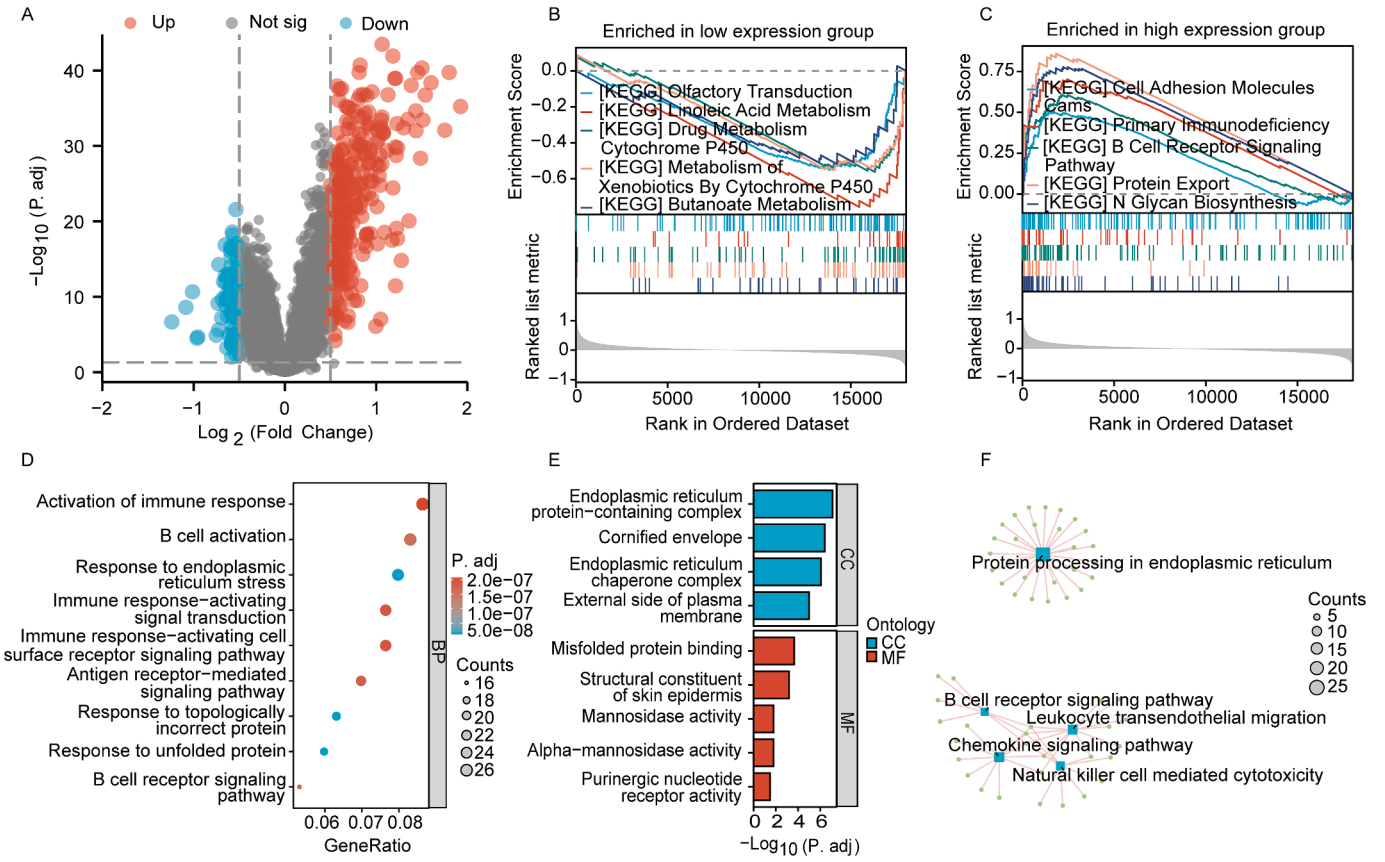

Fig. 5. Exploration of biological behavior related to PRDX4 in periodontitis. (A) Volcano plot showed differentially expressed genes (DEGs) based on PRDX4 expression. Red points indicated upregulated DEGs, blue points indicated downregulated DEGs, and grey points indicated DEGs with no significant differences in expression. **(B and C)** Gene set enrichment analysis (GSEA) of the significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the low- and high-expression groups of PRDX4. **(D-F)** Bar plot of enriched GO terms and enriched KEGG pathways analysis based on the expression of PRDX4.

inflammatory response in periodontitis was identified, providing a novel therapeutic strategy for periodontitis. More importantly, studies have provided strong evidence that mitochondria played a critical role in regulating B cell-mediated immune response (Demasi et al., 2013; Baracho et al., 2014). The specific mechanism underlying the mitochondrial-immune link in periodontitis needs further exploration.

We further identified PRDX4 and PDK1 as the main MIR-DEGs that regulate mitochondria-immune response links in periodontitis. We divided the periodontitis samples into low- and high-expression groups based on the levels of PRDX4 and PDK1. The functional analysis further revealed that the differential pathways between the low- and high-expression groups were considerably enriched in B cell activation, B cell receptor signaling pathway, immune response-regulating signaling pathway, and leukocyte mediated immunity and leukocyte migration. Furthermore, PRDX4 and PDK1 may also modulate other immune cells, including T cells, eosinophil, plasmacytoid dendritic cell, MDSC, monocyte, and natural killer cells, which needs further exploration.

PRDX4 (Peroxiredoxin-4) is a well-known antioxidant and a protective agent against various inflammatory and bone-destructive diseases (Tiedemann et al., 2019; Yamada and Guo, 2018). A study confirmed that PRDX4 played a central role in antibacterial immune modulation (Lipinski et al., 2019). To date, no study has explored the role of PRDX4 in periodontitis progression. Only one study indicated that the protein level of PRDX4 was upregulated in periodontal tissues from patients with periodontitis compared with that of healthy controls (Sunnetci-Akkoyunlu et al., 2023). Our results showed that the gene expression level of PRDX4 was higher in the clinical sample from patients with periodontitis than in those from healthy individuals. However, the gene expression level of PRDX4 in the periodontal tissues of mice was lower than that in control mice as compared to that of the control group. The most likely cause of the inconsistency between animal and clinical studies is as follows: First, basic scientific studies are traditionally conducted on cell lines and animal models, where it is impossible to completely reflect or replicate the in vivo clinical status (LaMoia and Shulman, 2021). Furthermore, periodontitis is a complex disease involving acute and chronic stages and different pathological mechanisms. The tissues might be collected from patients with different stages of periodontitis and therefore different gene expressions and regulations were involved (Tonetti et al., 2018). The animal models are hard to accurately and fully reflect the pathological state of periodontitis. Therefore, even inconsistent findings of PRDX4 between bioinformatic, clinical and in vivo data were detected, its roles in regulating periodontitis process are worthy of further exploration.

PDK1 is a protein-coding gene located in the mitochondrion matrix, which modulates mitochondrial function (De Rosa et al., 2021; Zeng et al., 2021). Furthermore, PDK1 is a critical regulator of immune responses and cells, including Th17 cells, Tregs, and NK cells (He et al., 2019; Gerriets et al., 2015). One study revealed the expression of PDK1 in the alveolar bone (Sima et al., 2016). However, the specific role of PDK1 in periodontitis progression remains elusive. In the present study, the bioinformatic results and expression from animal and clinical periodontal tissues all confirmed the increased expression of PDK1 in the periodontitis group compared with that in the control group. The increased expression of PDK1 gene level was also closely correlated with clinical probing pocket depth. Furthermore, PDK1 colocalized with B cells in both animal and clinical periodontal tissues, suggesting the possible role of PDK1 in regulating B-cell-mediated immune response in periodontitis. More studies are needed to investigate the role of PDK1.

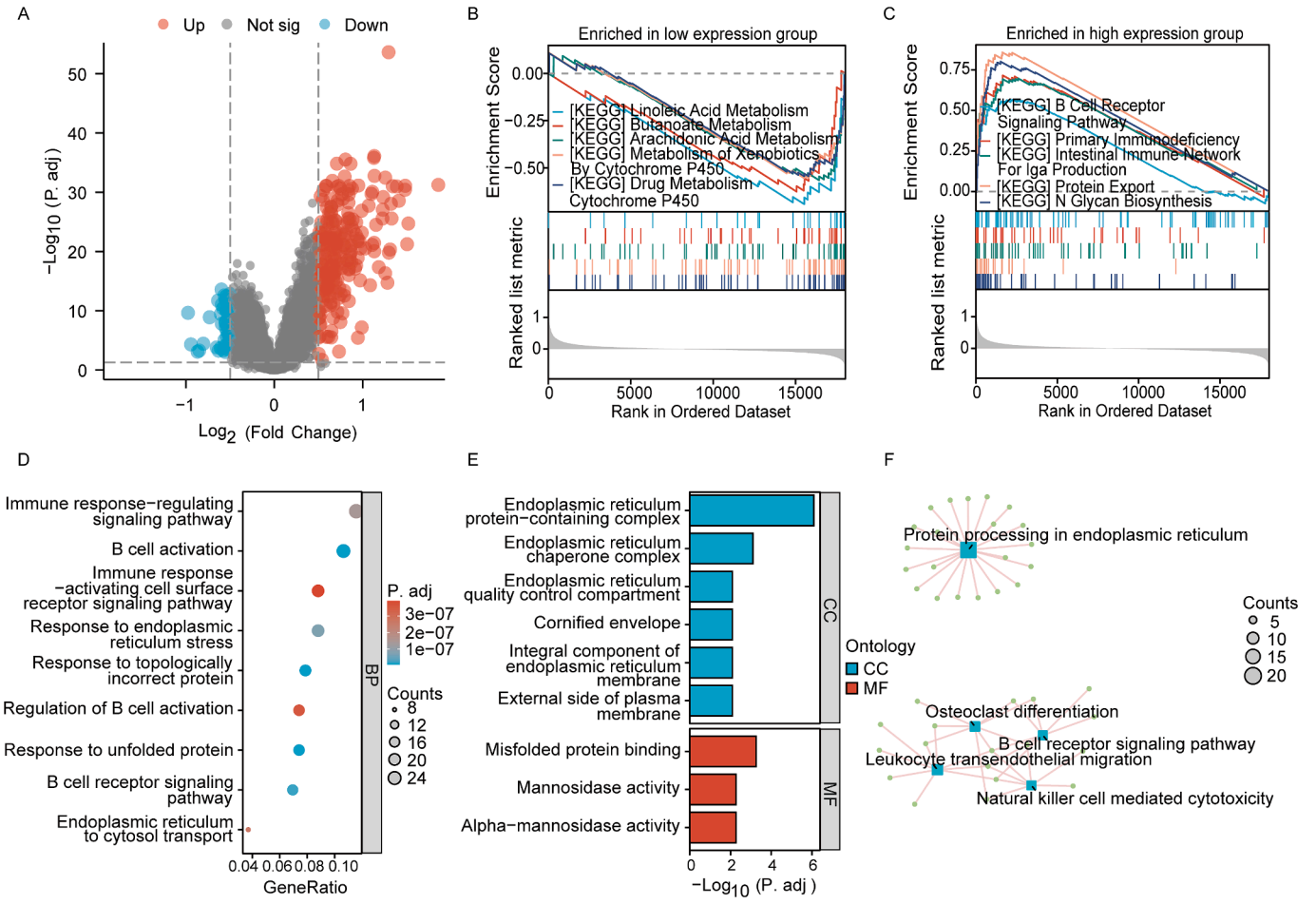

Fig. 6. Exploration of biological behavior related to pyruvate dehydrogenase kinase isoenzyme 1 (PDK1) in periodontitis. (A) Volcano plot showed differentially expressed genes (DEGs) based on PDK1 expression. Red points indicated upregulated DEGs, blue points indicated downregulated DEGs, and grey points indicated DEGs with no significant differences in expression. **(B and C)** Gene set enrichment analysis (GSEA) of significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the low- and high-expression groups of PDK1. **(D-F)** Bar plot of enriched Gene Ontology (GO) terms and KEGG pathway analysis based on the expression of PDK1.

In summary, mitochondria modulate the immune response in periodontitis, especially of B cells. PDK1 may be an essential regulatory gene linking mitochondrial events and immune response in periodontitis progression. This study provided novel insights into the regulatory mechanism of periodontitis and the potential application of PDK1 as a therapeutic target for mitochondrial immunotherapy.

5. Conclusions

Mitochondrial events play a key role in regulating immune response, especially of B cells, in periodontitis. The role of mitochondria in regulating B-cell-mediated immune response in periodontitis provides new insights into the pathogenic mechanisms and therapeutic strategies for periodontitis. PDK1 may be a regulatory factor linking mitochondrial events and B-cell mediated immune response in periodontitis and a potential therapeutic target for periodontitis.

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Institutional Review Board Statement.

The Ethics Committee of Wenzhou Medical University approved the experimental procedures (No. WYKQ2022019). Gingival tissues from 12 healthy subjects and 12 patients with severe periodontitis were obtained from the affiliated Stomatology Hospital of Wenzhou Medical University from January 2022 to August 2022. All the animal-related experiments had been approved by the Animal Ethics Committee of Wenzhou Medical University (WYDW2019-0665).

CRediT authorship contribution statement

Xiaoyu Sun: Writing – original draft, Data curation, Conceptualization. **Tong Wu:** Investigation, Formal analysis. **Zhan Yang:** Formal analysis, Data curation. **Shuhong Chen:** Formal analysis. **Zheyu Zhao:** Formal analysis. **Chaoming Hu:** Validation, Investigation. **Shengzhuang Wu:** Investigation. **Jiayu Wu:** Investigation. **Yixin Mao:** Investigation. **Jiefan Liu:** Investigation. **Chen Guo:** Supervision, Methodology. **Gang Cao:** Supervision, Methodology. **Xiangwei Xu:** Supervision, Methodology. **Shengbin Huang:** Writing – review & editing, Conceptualization. **Guang Liang:** Supervision, Conceptualization.

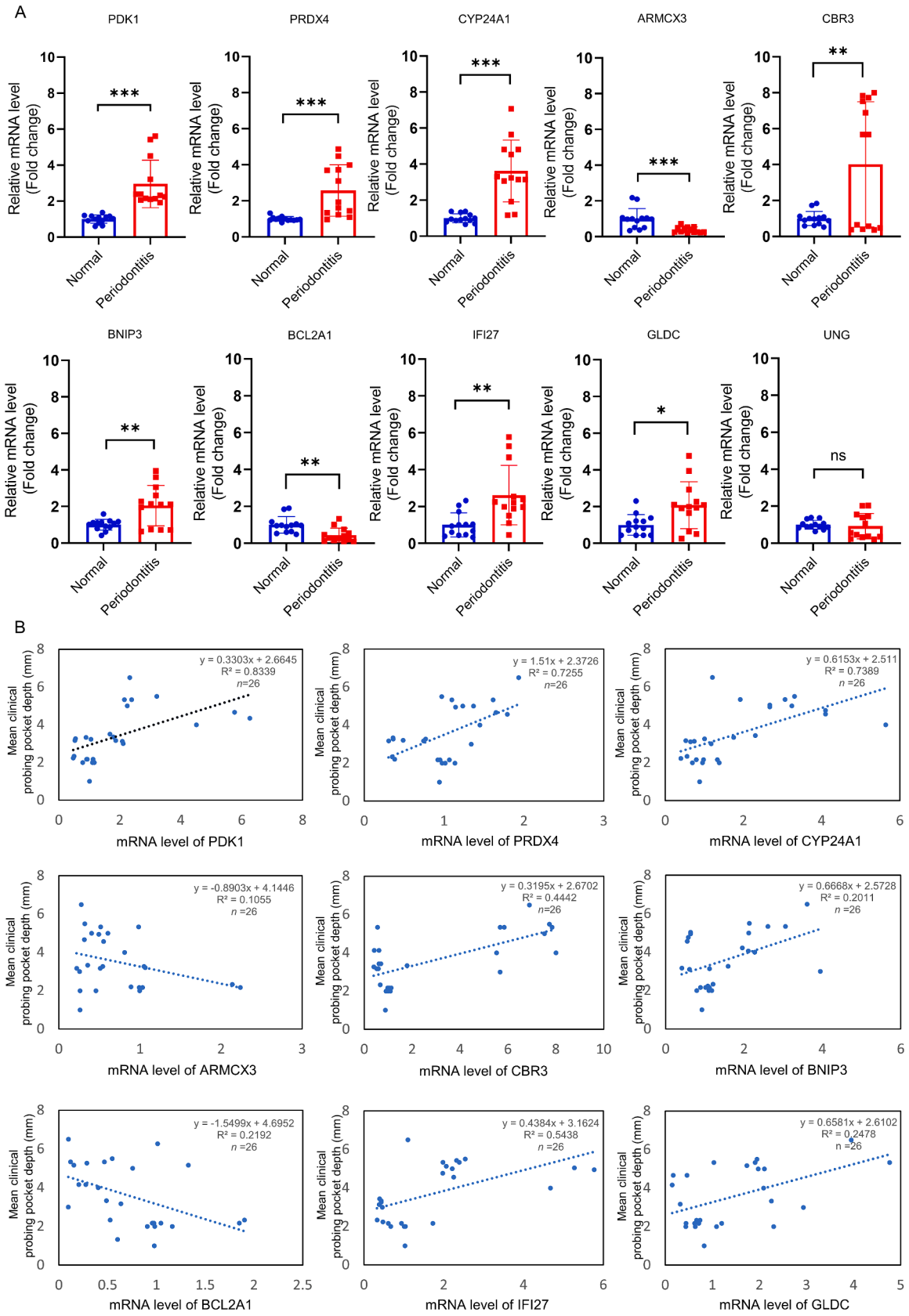

Fig. 7. Clinical validation of mitochondria-related differentially expressed genes (MIR-DEGs) and their correlation with clinical probing pocket depth. (A) The expression levels of MIR-DEGs in healthy and periodontitis tissue samples (n = 13). **(B)** Correlation between gene expressions of MIR-DEGs and the clinical probing pocket depth in periodontitis patients (n = 26). *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$; ns, non-significant.

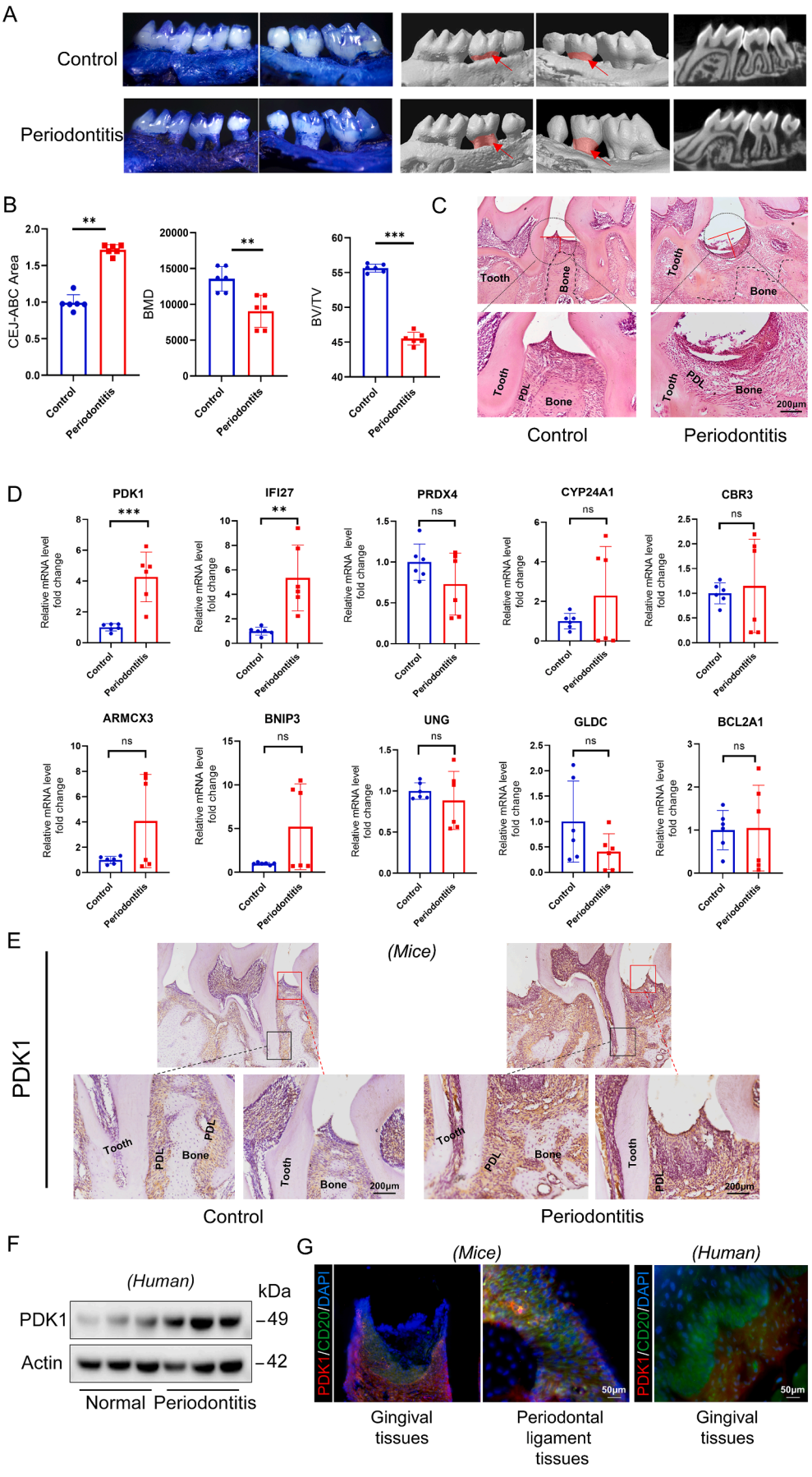

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Fig. 8. Verification of mitochondria-related differentially expressed genes (MIR-DEGs) in periodontal animal model. (A) Volume microscope and micro-computed tomography (μCT) images of mesial-distal bone of maxillary alveolar bone in control and periodontitis groups. Periodontitis-induced alveolar bone loss was quantified by measuring the area from the cemental-enamel junction (CEJ) to the alveolar bone crest (ABC) on the buccal and interdental root surfaces around the second molar (the red linear area). (B) Bone-related parameters, including CEJ-ABC area, bone mineral density (BMD), and bone value/total value (BV/TV), were quantified. (C) Representative hematoxylin and eosin stained images of gingival tissues after ligature treatment. PDL (periodontal ligament) (n = 6). (D) The expression levels of MIR-DEGs between the control and periodontitis groups (n = 6). (E) Representative images from PDK1 immunohistochemical staining of mouse periodontal sections. (F) Western blot band of PDK1 protein expression in clinical gingival tissues from healthy individuals and patients with periodontitis. (G and H) PDK1 (red) and B cells (CD20, green) were stained in alveolar bone sections of the second maxillary molars of mice and human gingival tissues. DAPI was used to counterstain DNA. PDL, periodontal ligament tissues.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2024.148476.

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