

Novel Insights from Comprehensive Bioinformatics Analysis Utilizing Large-Scale Human Transcriptomes and Experimental Validation: The Role of Autophagy in Periodontitis

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Objective: Autophagy plays a crucial role in the pathophysiology of periodontitis, yet its precise involvement in the disease process remains elusive. The aim of the present study was thus to investigate the involvement of autophagy in the pathology of periodontitis. This investigation involved transcriptomic analysis of a broad range of human samples and complemented by in vitro experimentation.

Materials and Methods: We analyzed the transcriptomes of human gingival tissues from individuals with periodontitis and health controls to identify the differential expression of autophagy-related genes (DEARGs) and to investigate their potential interactions and functional pathways. Additionally, protein-protein interaction (PPI) networks were constructed to identify key functional modules and hub genes. Experimental validation of autophagy regulation in periodontitis and identification of key autophagy-regulating genes was accomplished through in vitro cellular experiments. Subsequently, a comprehensive analysis of immune cell infiltrate utilizing the CIBERSORT algorithm was performed. Finally, leveraging the DSigDB database, potential candidate drugs for periodontitis treatment targeting autophagy were predicted.

Results: A total of 79 genes have been identified as DEARGs in periodontitis. An intricate interplay among the DEARGs and their impact on the regulatory mechanisms of autophagy within the context of periodontitis was observed. Subsequently, 10 hub genes were discerned through the establishment of a PPI network. Furthermore, dysregulated autophagic activity in periodontitis was validated, and 9 key genes (APP, KDR, IL1B, CXCL12, CXCR4, IL6, FOS, LCK, and SHC1) were identified through in vitro experiments. Our analysis unveiled an association between these genes and altered immune cell infiltration in periodontitis. Additionally, we predicted potential therapeutic agents such as curcumin, 27-hydroxycholesterol, and Trolox, showing promise in the treatment of periodontitis by modulating the autophagic process.

Conclusion: This study identified nine key genes for autophagy regulation and potential therapeutic agents in periodontitis. These findings not only enhance our comprehension of the pathological mechanisms of periodontitis but also provide substantial evidence for the advancement of novel therapeutic strategies.

Keywords: periodontitis, transcriptome, autophagy, key genes, diagnosis, database

Introduction

Periodontitis, a prevalent chronic oral inflammatory disease, is characterized by the pathological loss of tooth-supporting tissues like the gingiva and alveolar bone.¹ Its progression can lead to compromised mastication and tooth loss.² Additionally, numerous studies have indicated periodontitis as a relevant risk factor for a range of non-oral health conditions, encompassing cardiovascular disease, diabetes, and kidney injury, highlighting its systemic health implications.¹ Nonetheless, the etiology of periodontitis remains enigmatic, with current diagnostic practices

predominantly relying on clinical and radiographic assessments. These methods offer limited therapeutic direction, contributing to treatment delays or excessive medication in a considerable proportion of cases.^{3–5} Advances in molecular biology have paved the way for tailored and precise management of periodontitis, and the associated precise diagnostic modalities and targeted therapies may play a crucial role in future periodontal care.^{4,6,7} Therefore, a thorough investigation into the molecular biology aspects of periodontitis pathogenesis is imperative for comprehensive understanding and effective intervention.

Autophagy, a highly conserved and meticulously regulated physiological process, serves as an important regulatory mechanism for maintaining cellular homeostasis and organismal balance.⁸ In general, autophagy clears detrimental cytosolic components via lysosomal degradation pathways, safeguarding against cellular harm and fostering survival. Conversely, the breakdown of autophagy compromises this protective function.^{9–11} Although autophagy has been intensively studied, excessive activation of autophagy, as a distinct form of autophagic dysregulation, participates in the advancement of numerous diseases.¹² Excessive autophagic activation results in the degradation of crucial cellular components and the dysfunction of organelles, which may ultimately lead to autophagic cell death.^{11,13–15}

Autophagy-related genes (ARGs) are pivotal in governing the autophagic pathway.^{16,17} The significant regulatory influence of ARGs in inflammatory conditions has been substantiated,^{18,19} A dual role of autophagy in periodontitis has been observed.^{20–22} The upregulation of LC3B, serving as a marker for autophagy, has been observed in human periodontitis gingival tissue and experimental models, with contributory factors like dental plaque instigating atypical autophagy in diverse gingival cells, thereby promoting periodontitis development.^{23–25} Conversely, autophagy may counter apoptosis-induced cell death, preserving cell viability in periodontal diseases, and inadequate autophagic responses could heighten inflammation and susceptibility to periodontitis.^{26,27} Considering the crucial yet debated implications of autophagy in periodontitis, further investigations are warranted to elucidate the exact functional role of autophagy.^{28,29}

The aim of this study is to examine the function and pathological mechanisms of autophagy in periodontitis using transcriptomic data from a large cohort of human samples and *in vitro* experiments.

Materials and Methods

Ethics Approval and Consent to Participate

The study protocol for this study was approved by the Ethics Committee of The Affiliated Stomatological Hospital, Jiangxi Medical College, Nanchang University (Approval No. 2022016).

The Collection and Processing of Data

In this study, we downloaded the GSE16134 microarray dataset from the NCBI-GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). The study involved transcriptomic analysis of gingival tissues from 120 patients diagnosed with moderate to severe periodontitis. The ID conversion process is based on the platform file provided by the original researchers in the GSE16134 dataset. This involves transforming the original IDs directly into gene symbols. In cases where multiple IDs correspond to the same gene symbol, a strategy of taking the median of the expression levels associated with these IDs is adopted to ensure the accuracy and representativeness of the data.

Identifying Differentially Expressed Genes (DEGs) and Differentially Expressed ARGs (DEARGs)

Utilizing the limma package (version 3.48.0) in the R software (version 4.1), we identified DEGs in the gingiva of periodontitis patients. The moderated *t*-test from the limma package was employed for statistical assessment, with the results subsequently adjusted using the Benjamini-Hochberg method. Prior to analysis, the data underwent preprocessing via quantile normalisation. The boxplots before and after standardization are presented in [Figure S1](#). Subsequently, DEGs meeting the criteria of $|\log_2FC| \geq 0.5$ and the false discovery rate (FDR) < 0.05 were selected.^{30,31} Autophagy-related genes (ARGs) were identified utilizing resources such as the Autophagy Database (<http://www.tanpaku.org/autophagy/index.html>),³² the Human Autophagy Database (<http://www.autophagy.lu/index.html>),³³ and the Human Autophagy Modulator Database (<http://hamdb.scbdd.com>).³⁴ After

removing duplicate genes, a merged gene set comprising 1174 ARGs was established, as shown in [Table S1](#). For the extraction of DEARGs from the DEGs, the Venn diagram package in the R software was employed.

Gene Set Enrichment Analysis (GSEA)

We conducted GSEA utilizing the GSEA software (version 3.0.0) (<http://software.broadinstitute.org/gsea/index.jsp>), based on phenotypic data, to explore the potential link between ARGs and human gingiva in periodontitis.³⁵ A gene set comprising 1174 ARGs was employed as previously described. Genes from the GSE16134 dataset were quantitatively assessed and ranked according to their respective expression levels, enabling the calculation of an enrichment score (ES). Significance in GSEA was defined by an FDR value of less than 0.05.

Comprehensive Enrichment Analyses of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

The DEARGs were subjected to GO and KEGG enrichment analyses utilizing the ClusterProfiler package (version 3.12.0) in R software. GO (Gene Ontology) functional analysis encompasses three domains: Cellular Component (CC), Molecular Function (MF), and Biological Process (BP). Enrichment results with an FDR below 0.05 were considered statistically significant.

The Construction and Analysis of Protein-Protein Interaction (PPI) Networks

The PPI network of DEARGs was established and visualized utilizing the STRING database (<https://string-db.org/>) and the Cytoscape software (version 3.8.2).^{36,37} To identify the functional key module, we employed the Cytoscape plugin MCODE (degree cutoff = 2, node score cutoff = 0.2, K-core = 2, and max depth = 100). Using Cytohubba's built-in MCC algorithm, hub genes were determined by evaluating each gene within the PPI network and ranking them based on their values. These 10 top-ranked genes were recognized as hub genes.

Culture of Primary Human Gingival Fibroblasts (HGFs)

HGFs were purchased from Wuhan Procell Life Science and Technology Co., Ltd. (Wuhan, China). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (H-DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fresh fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (Solaibao, Beijing, China). The cell culture process occurred in an incubator, where the cells were exposed to 5% CO₂, 21% O₂, and a temperature of 37°C. Subsequent experiments utilized cells derived from the 4th to 6th generation.

We established an in vitro periodontitis model by exposing HGFs to lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, USA). Inhibition of autophagy was achieved through the utilization of 3-methyladenine (3-MA) (MedChemExpress, NJ, USA).²⁴ In the LPS-induced periodontitis model, HGFs were subjected to a 24-hour treatment with 10 µg/mL LPS. Prior to LPS treatment, cells in the LPS + 3-MA group were pre-treated with 3-MA (5 mM) for an additional 24 h. The control group received only the respective solvent.

Cytotoxicity Assays

To assess cytotoxicity, the lactate dehydrogenase (LDH) activity was quantitatively measured employing a specific LDH assay kit (Beyotime, Shanghai, China). HGFs were first seeded into a 96-well plate following different treatments. Subsequently, the supernatant was incubated with the active solution of the LDH assay for 30 min at a temperature of 37°C. The absorbance was then quantified at 490 nm utilizing the Spark R microplate reader. Finally, cytotoxicity was determined using the formula provided in the assay instructions. Cytotoxicity or Mortality (%) = (Absorbance of Treated Sample - Absorbance of Sample Control Well) / (Absorbance of Maximum Cellular Enzyme Activity - Absorbance of Sample Control Well) × 100.

Quantitative Real-Time PCR (qRT-PCR)

After treating the HGFs according to the aforementioned protocol, total RNA extraction was carried out. Subsequently, the RNA concentration was accurately determined, and reverse transcription into cDNA was performed employing the

AidMM reverse transcription kit (ThermoFisher Scientific, Massachusetts, USA). qRT-PCR analysis was executed utilizing SYBR Premix EX Taq (Takara, Japan) on the StepOnePlus PCR System (Applied Biosystems, CA, USA). Normalization of the mRNA expression levels was conducted using ACTB as a reference, with calculations performed according to the $2^{-\Delta\Delta CT}$ method. The sequences of the primers used for the detection of the expression patterns of the genes are detailed in [Table S2](#).

Western Blot

Cells were subjected to lysis using RIPA lysis buffer (Beyotime, Shanghai, China), supplemented with 1% phenylmethylsulfonyl fluoride (PMSF). The lysates were then centrifuged for 15 min. Following centrifugation, the supernatant was divided into two portions. One portion was utilized for protein quantification using a BCA kit, while the other part was prepared for further analysis by mixing with loading buffer and boiling for denaturation.

After being separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were migrated onto a polyvinylidene fluoride (PVDF) membrane for subsequent analysis. The membranes were blocked utilizing 5% skim milk powder for 2 h, followed by an overnight incubation at 4°C with primary antibodies specific to LC3 (Proteintech, 14600-1-AP, Wuhan, China) and β -actin (Biosharp, Hefei, China). Following three washes with Tris-buffered saline containing Tween (TBST), the membranes were incubated for 2 h with the appropriate secondary antibodies (Beyotime, Shanghai, China).

Following additional TBST washes, the blots were developed for detection using an electrochemical luminescence (ECL) kit (Beyotime, Shanghai, China).

Immunofluorescence Analysis

The HGFs were cultured in 24-well plates and treated as described previously. Following treatment, the cells were immobilized by fixing with a 4% paraformaldehyde solution for 10 min. After fixation, the cells underwent a 30-minute blocking with 5% bovine serum albumin (BSA) in PBS at 37°C, followed by an overnight incubation at 4°C with an anti-LC3 primary antibody (Proteintech, 14600-1-AP, Wuhan, China). In the dark, the cells were incubated with a secondary antibody (Beyotime, China) for 1 h, followed by staining of their nuclei with 4',6-diamidino-2-phenylindole (DAPI) for 10 min under the same conditions. Images were captured employing a fluorescence microscope (Nikon, Tokyo, Japan), with appropriate excitation wavelengths.

Imaging by Transmission Electron Microscopy (TEM)

After centrifugation and culture medium removal, the cells were fixed with 2.5% glutaraldehyde at 4°C. Following the initial fixation, the samples were further fixed with 1% osmic acid at room temperature for 2 hours. The samples then underwent a dehydration process in a series of alcohol solutions, followed by embedding.

The resulting resin blocks were sectioned into ultra-thin slices of 60–80 nanometers in thickness using an ultramicrotome, and these slices were picked up on 150-mesh formvar-coated copper grids. Subsequently, the copper grid-mounted slices were stained with 2% uranyl acetate and 2.6% lead citrate. Finally, autophagosomes and autolysosomes were observed and imaged using a transmission electron microscope (Hitachi 7800, Japan).

Immune Infiltration Analysis

Employing the CIBERSORT algorithm (Version released in 2015), we quantified the proportions of 22 distinct immune cell types present in the sample, thereby enabling a comprehensive analysis.³⁸

Subsequently, the correlation between hub genes and differentially infiltrating immune cells (DIICs) was analyzed employing a linear regression analysis. The data utilized for analysis are identical to the standardized data employed for differential analysis.

Predicting the Potential of Therapeutic Drugs

Interaction data between proteins and drugs were obtained from the DSigDB database (Version released in 2015) (<http://dsigdb.tanlab.org/>) to predict potential drugs capable of modulating autophagy for the treatment of periodontitis.³⁹ The prediction process involved applying specific criteria, including a stringent cutoff of FDR <

0.001 and a combined score exceeding 1000. A lower FDR value indicated a more robust correlation between the drugs and their target proteins. Identification of the chemical structures of the predicted drugs was achieved through the use of the Drugbank (<http://www.drugbank.ca>) and PubChem databases (<http://pubchem.ncbi.nlm.nih.gov>).

Statistical Analysis

The GraphPad Prism software (version 8.0.2) (La Jolla, CA, USA) was employed to carry out the statistical analyses. Group comparisons were conducted utilizing t-tests or signed rank tests, based on the data characteristics. In cases involving multiple groups, differences were assessed using an ordinary one-way analysis of variance (ANOVA). Following the ANOVA results indicating statistical significance, subsequent post-hoc tests were performed to identify specific group differences. Spearman correlation test was utilized for correlation analyses to evaluate relationships between variables. Data are presented as mean values with standard deviation (SD).

Results

Comprehensive Protocol of This Study

Figure 1 illustrates the comprehensive design of the study. Transcriptome data from a total of 310 human gingival samples were included in our study, comprising 241 samples from periodontitis gingival tissues and 69 from healthy controls. Prior to subsequent analysis, all raw expression data underwent normalization following the methodology described above.

Identification of DEG and DEARGs

GSEA is a classic and widely used method for assessing the overall correlation between a set of signature genes and the disease transcriptome. GSEA was conducted using dataset GSE16134 to investigate the variation in ARGs within the human periodontitis transcriptome. The results, depicted in Figure 2A, demonstrated a substantial positive correlation of

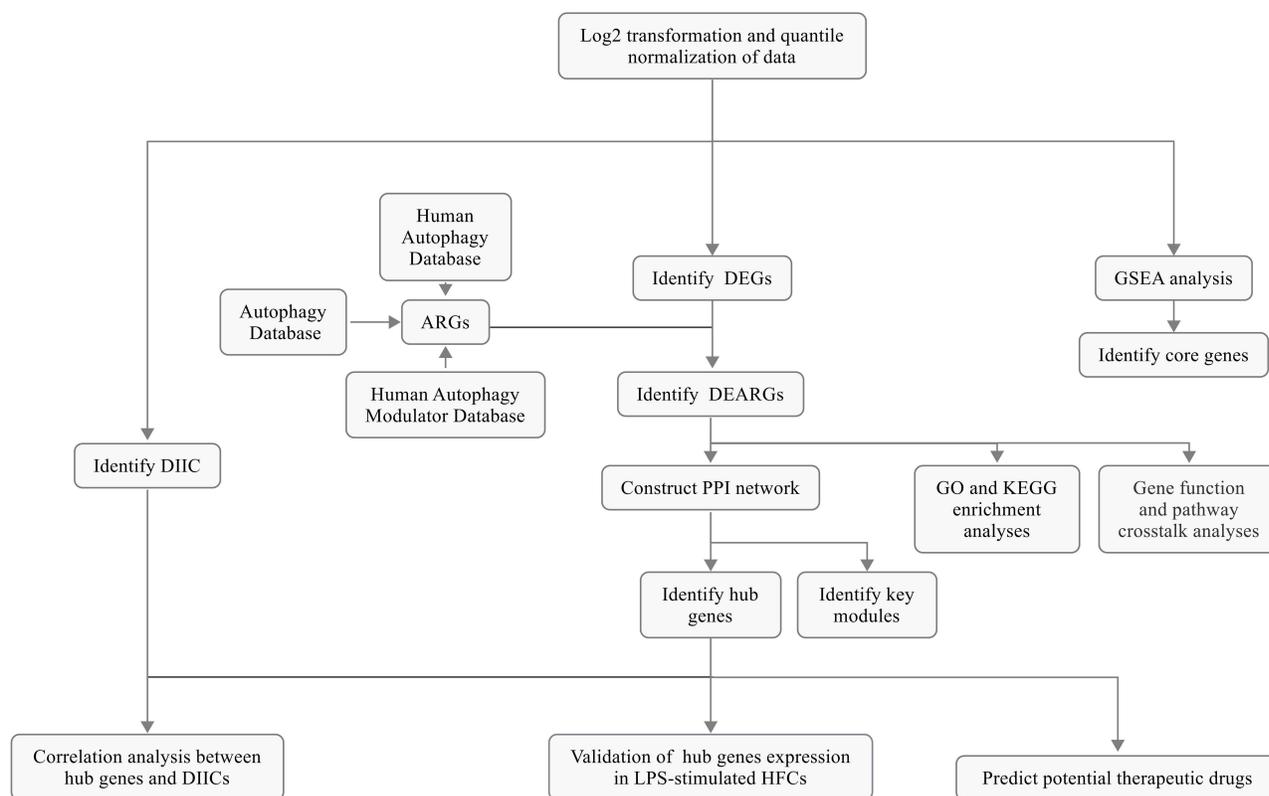


Figure 1 Schematic representation of the overall study protocol.

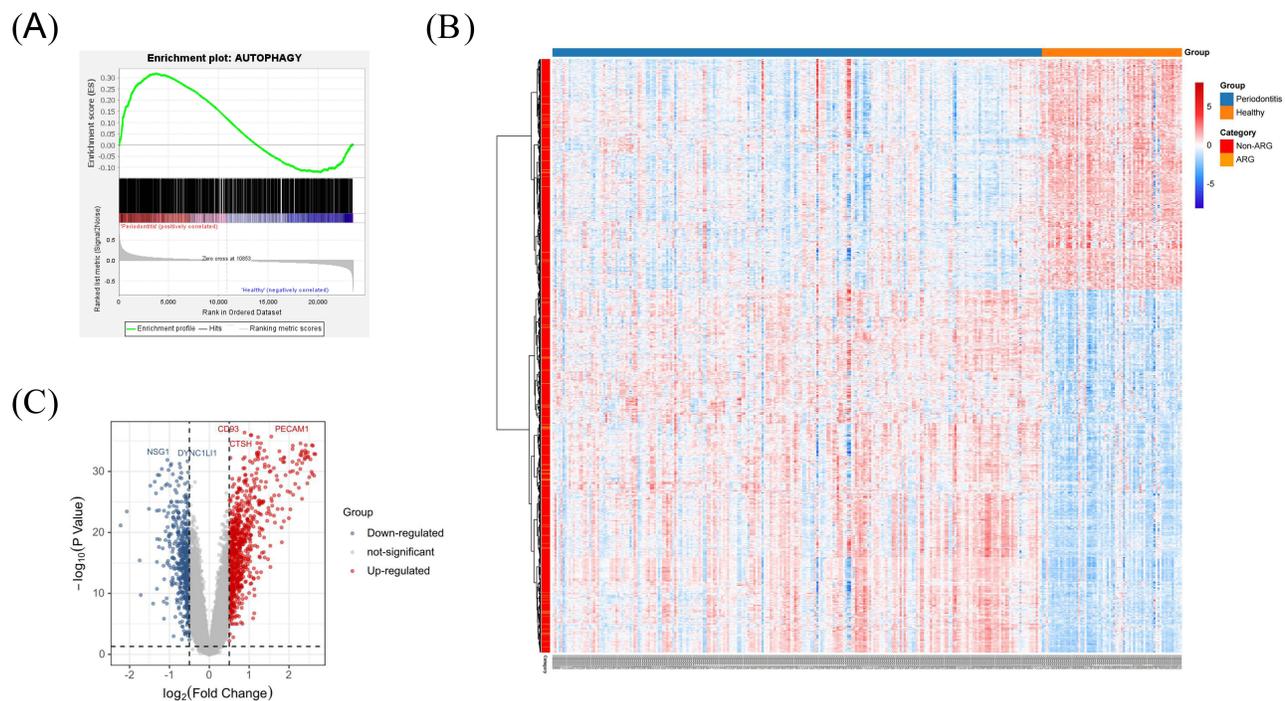


Figure 2 Gene set enrichment analysis (GSEA) and identification of differentially expressed genes (DEGs). **(A)** GSEA on the autophagy-related genes (ARGs) set identified from GSE16134; **(B and C)** Clustered heatmap **(B)** and volcano plot **(C)** of the DEGs identified from GSE16134 (FDR < 0.05 and $|\log_2FC| \geq 0.5$).

the ARGs set in periodontitis gingiva compared to healthy gingiva (Normalized Enrichment Score (NES) = 1.468, FDR = 0.027). This finding underscores the widespread dysregulation of ARGs as a key characteristic in the altered transcriptome of human periodontitis gingiva. The data derived from the human gingival transcriptome establish a significant association between autophagy and periodontitis.

Our transcriptomic analysis identified 1279 DEGs (782 upregulated, 497 downregulated) in periodontitis samples compared to controls (Figure 2B and C). Notably, 79 ARGs overlapped with these DEGs, signifying their importance in periodontitis (Figure 3A–C and Table S3).

GO and KEGG Analysis of DEARGs

We investigated the functions and associated pathways of DEARGs through GO and KEGG enrichment analyses. These DEARGs are implicated in regulating crucial biological processes (BP), including lymphocyte differentiation, T cell activation, peptidyl-tyrosine phosphorylation, peptidyl-tyrosine modification, positive regulation of cell adhesion, T cell differentiation, and neutrophil homeostasis (Figure 4A). Considering the established connections between autophagy and these specific BPs in a variety of biological contexts,^{40,41} it is conceivable that autophagy may initiate or influence these processes in the context of periodontitis, indicating a potential interplay between autophagy dysregulation and BP modulation in the pathophysiology of periodontitis. Regarding their cellular components (CCs), the DEARGs were predominantly localized in the membrane region, endocytic vesicle, and membrane raft (Figure 4B). With regard to their molecular functions (MFs), the DEARGs demonstrated enrichment in protein serine/threonine kinase activity, cytokine receptor binding, and growth factor receptor binding. (Figure 4C). The KEGG pathway analysis highlighted the involvement of DEARGs in autophagy, the chemokine signaling pathway, and rheumatoid arthritis. (Figure 4D).

Previous studies have unveiled reciprocal crosstalk between these pathways or functions and the modulation of autophagy in diverse disease processes, with some being recognized as regulators of autophagy in the context of periodontitis.²³ Consequently, our subsequent analysis delved into exploring the interplay between these genes and various pathways or functions. The results indicate the presence of intricate interactions between DEARGs and associated functions or pathways, with a significant impact on the biological function of autophagy and, consequently,

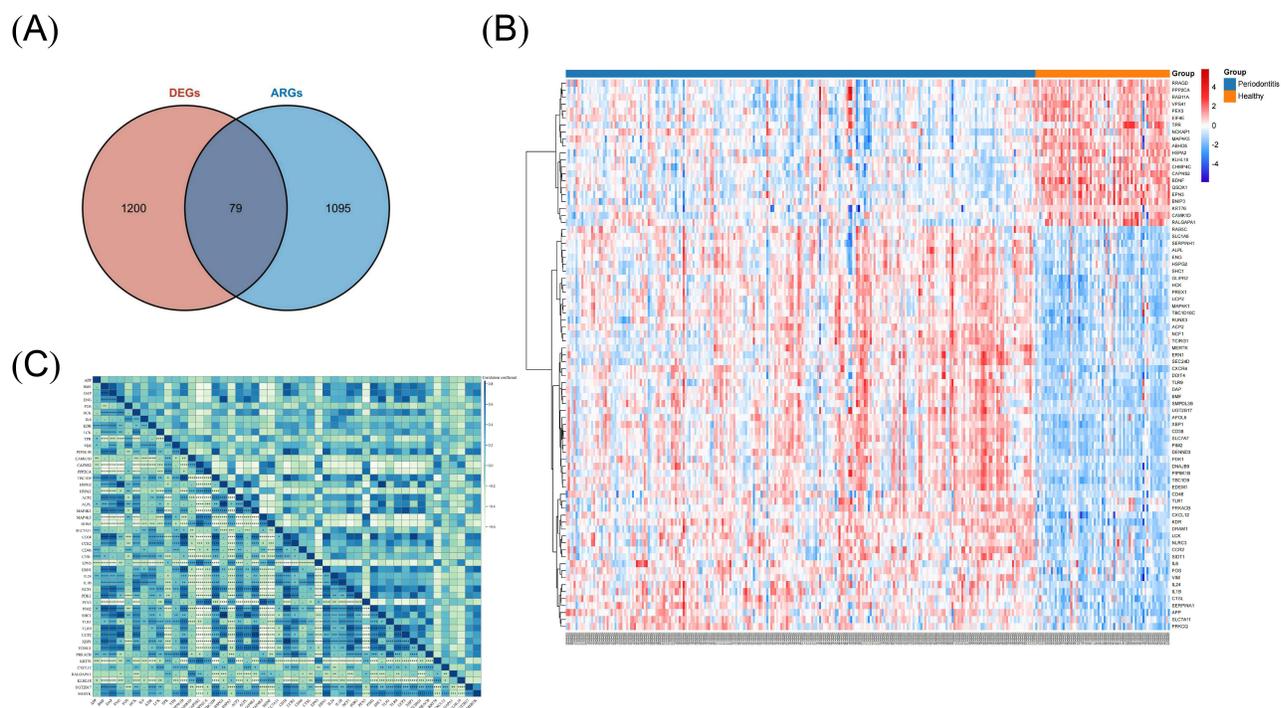


Figure 3 Identification of differentially expressed autophagy-related genes (DEARGs). **(A)** Venn diagram representing the overlap between the differentially expressed genes (DEGs) identified from GSE16134 and autophagy-related genes (ARGs) in autophagy-related databases; **(B and C)** The cluster heatmap **(B)** and the correlation heatmap **(C)** of DEARGs identified from GSE16134.

on the pathological process in periodontitis (Figure 4E–H). This intricate network of interactions emphasizes the multifaceted involvement of autophagy dysregulation in the pathophysiology of periodontitis, suggesting the potential therapeutic targets that could be harnessed to modulate autophagy and ameliorate the disease process.

The Generation and Analysis of a PPI Network

We have used the construction of a PPI network to elucidate the complex relationship between DEARGs and functions or pathways. Utilizing the STRING database, we were able to establish a PPI network and identified functional modules and hub genes through the Cytoscape software (Figure 5A). By applying the MCODE plugin, we revealed one key gene module (Figure 5B). Subsequently, employing the MCC algorithm within the Cytohubba plugin, we ranked the top 10 genes (CXCR4, CD38, FOS, CXCL12, IL1B, KDR, IL6, LCK, APP, SHC1) as hub genes (Figure 5C). The distinct expression patterns of these hub genes in periodontitis gingiva are visually represented in Figure 5D, illustrating their differential expression within this pathological framework. Figure 5E shows interactions among hub genes, while Figure 5F demonstrates the large number of connections between hub genes and other DEARGs, shedding light on the complex interplay within the regulatory network associated with autophagy dysregulation in periodontitis.

Validation by in vitro Experiments

To study the autophagy levels and expression of hub genes in periodontitis, an in vitro periodontitis model using LPS-treated HGFs was implemented. Figure 6A demonstrates a significant increase in cytotoxicity in the LPS-induced periodontitis model, while the 3-MA autophagy inhibition group showed a decrease in cytotoxicity. As shown in Figure 6B and C, LPS treatment led to a substantial elevation in the LC3-II/LC3-I ratio, a pivotal functional marker of autophagic flux, while treatment with 3-MA alleviated this ratio. Furthermore, immunofluorescence analysis showed an augmentation of LC3-positive puncta in the LPS-induced periodontitis model compared to the control group. These autophagic characteristics were markedly suppressed by 3-MA. Notably, Transmission Electron Microscopy (TEM) analysis further confirmed these findings (Figure 6D and E).

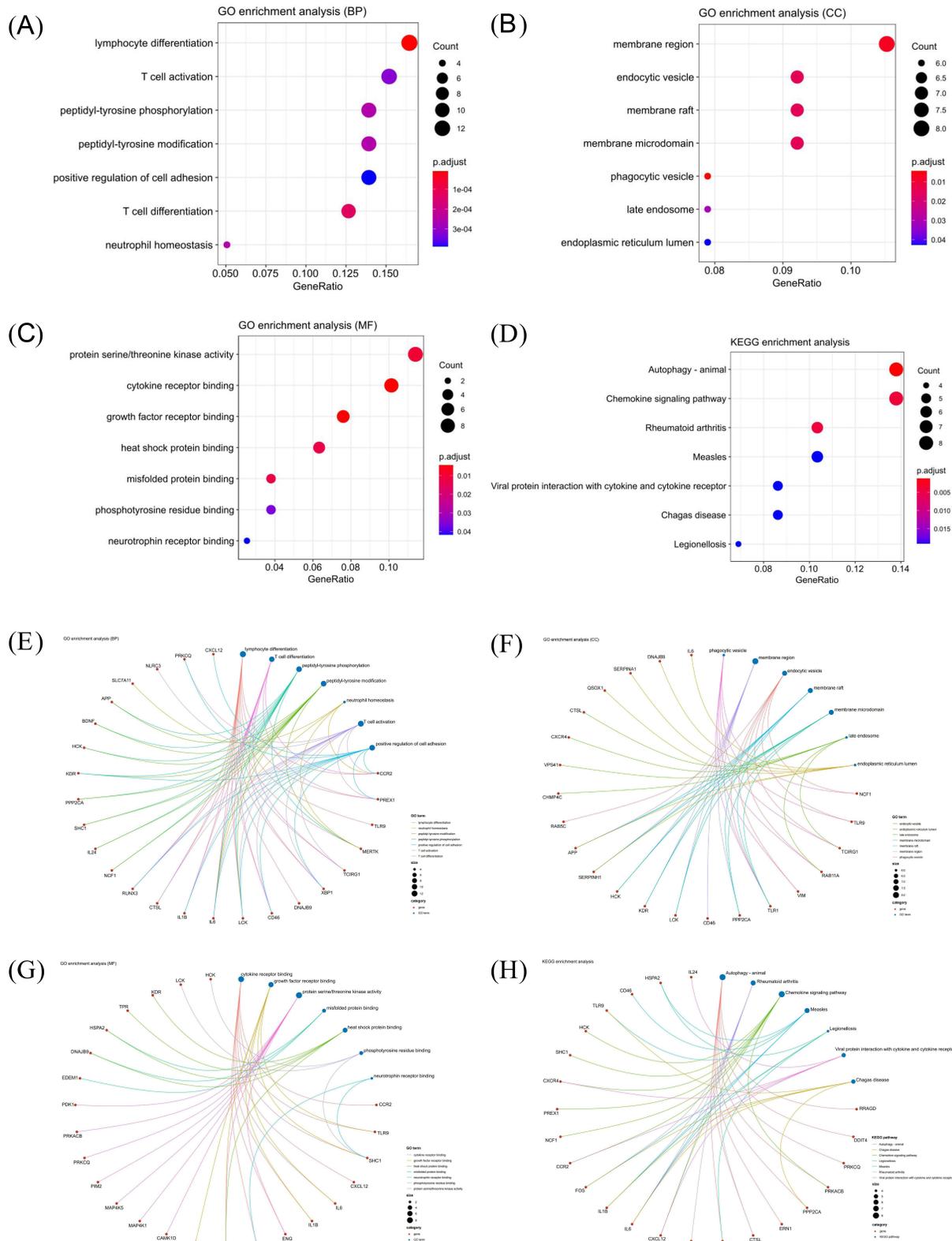


Figure 4 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of differentially expressed autophagy-related genes (DEARGs). **(A–C)** GO enrichment analysis in the biological process category (BP) **(A)** the cellular component category (CC) **(B)** and the molecular function category (MF) **(C and D)** KEGG enrichment analysis of DEARGs; **(E–G)** Interaction between DEARGs and the biological functions in BP **(E)**, CC **(F)** and MF **(G and H)** Interaction between DEARGs and KEGG pathways.

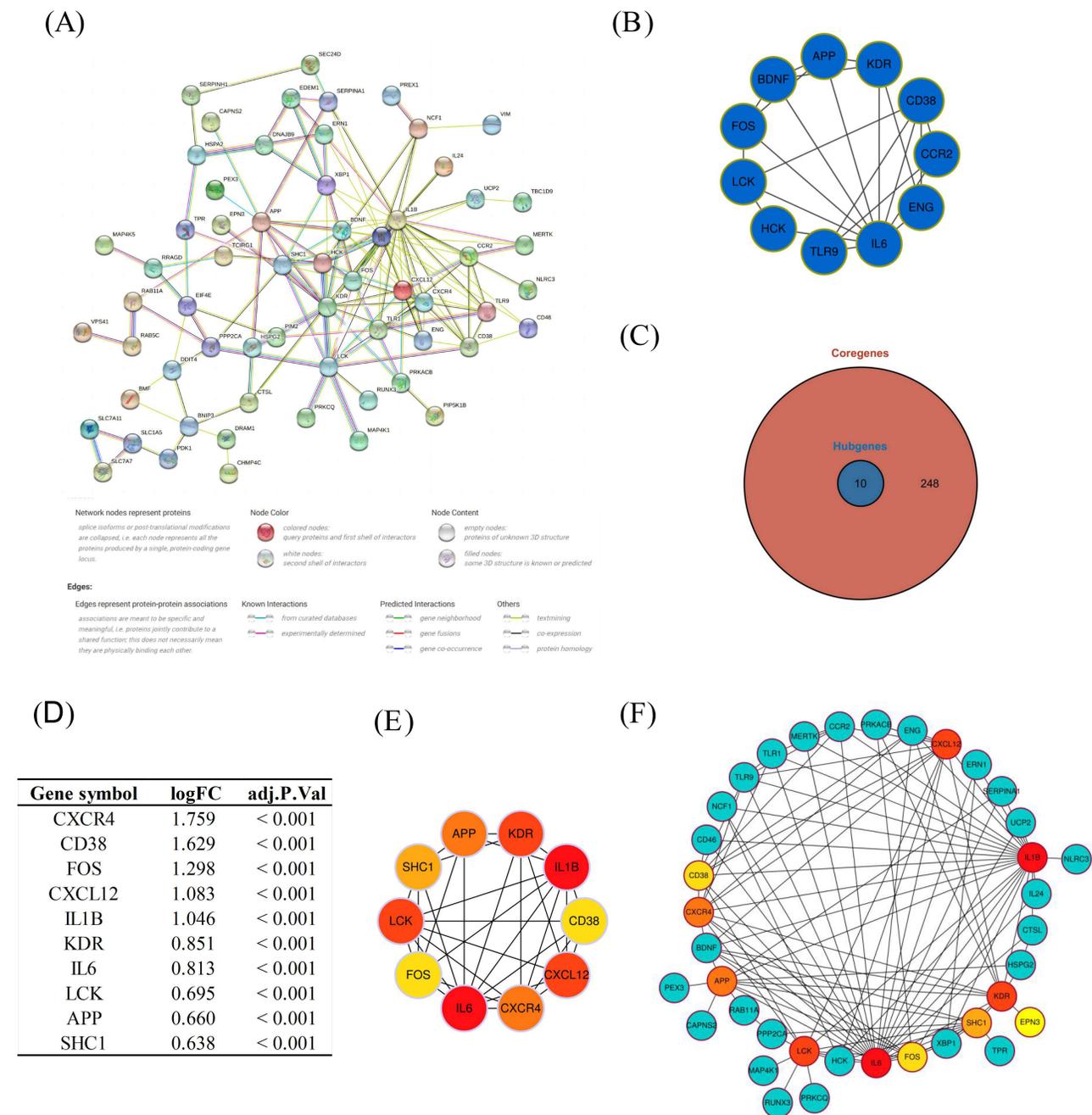


Figure 5 Protein-protein interaction (PPI) networks and identification of key module and hub genes. (A) PPI network of differentially expressed autophagy-related genes (DEARGs); (B) The key gene module identified by the MCODE plugin; (C) The top 10 genes identified as hub genes; (D) the distinct expression patterns of hub genes; (E) Interactions among hub genes; (F) Connections between the hub genes and the DEARGs.

The results from both the in vitro experiments and transcriptome analysis of human samples demonstrated dysregulation of autophagy levels in periodontitis. Subsequent studies were conducted to assess the level of expression of the hub genes in vitro experiments. The results of the qRT-PCR experiments showed that there was a significant increase in the expression of these hub genes after treatment with LPS. This trend was notably mitigated by 3-MA pretreatment, except for CD38, indicating that they are involved in autophagy regulation in periodontitis. Therefore, it was determined that the nine genes (CXCR4, FOS, CXCL12, IL1B, KDR, IL6, APP, LCK, and SHC1) from the original ten hub genes were the key genes (Figure 6F).

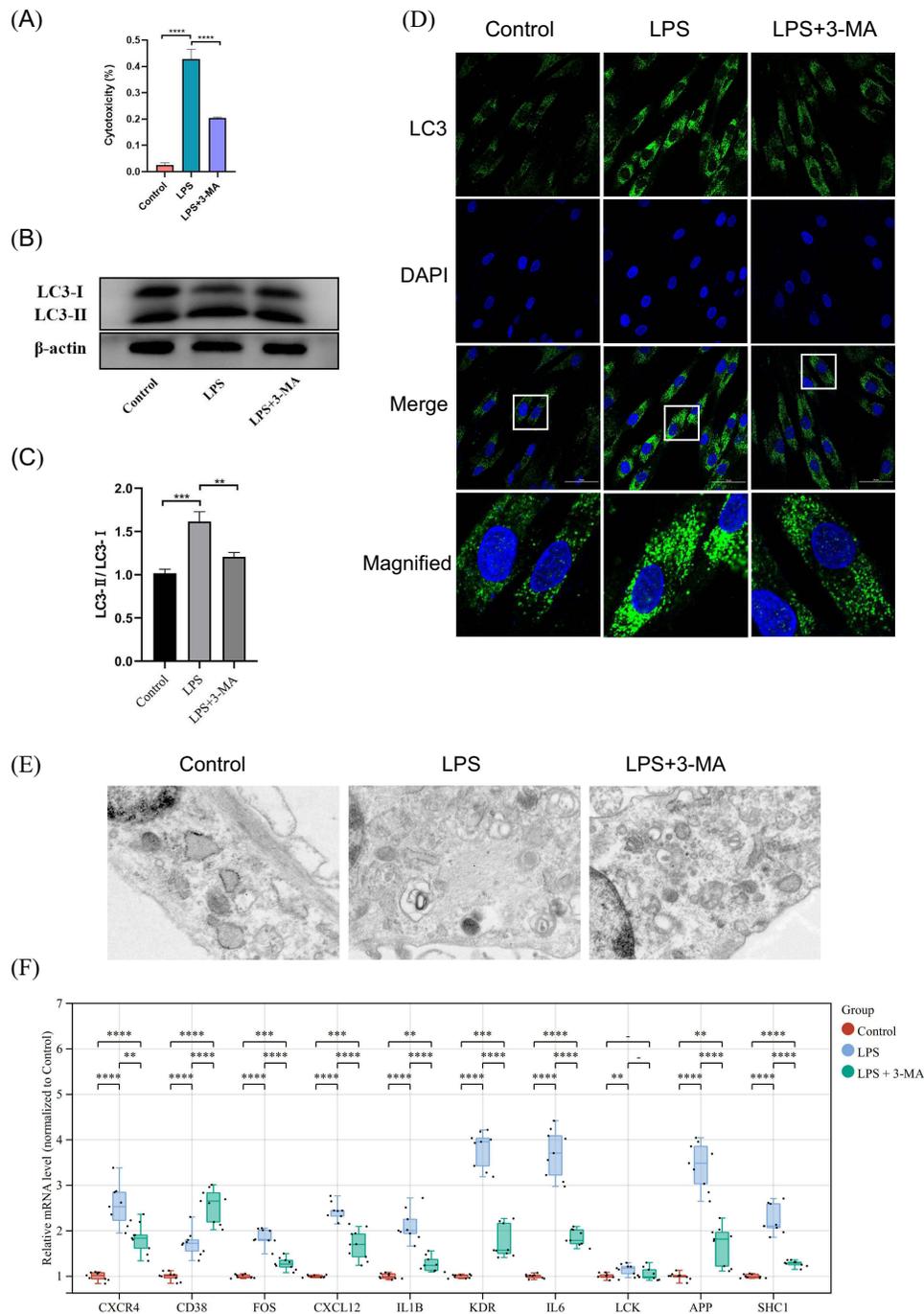


Figure 6 In vitro experimental model of periodontitis confirming the presence of autophagy dysfunction and identification of nine key genes. An in vitro model of periodontitis was established by treating HGFs with LPS. Autophagy inhibition was achieved by utilizing 3-MA. HGFs were divided into 3 groups for the in vitro study: the control group, the LPS-induced periodontitis group, and the LPS + 3-MA group. **(A)** Cytotoxicity test; **(B and C)** Western blot analysis; **(D)** Immunofluorescence analysis; **(E)** TEM analysis; **(F)** Verification of the transcript levels of the hub genes by qRT-PCR. Data are expressed as mean \pm SD. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

The results of this study further confirm the extensive presence of autophagic dysregulation in periodontitis (Figure 6B–E), indicating that autophagy may impact the pathophysiological processes of periodontitis by regulating the functions of these key genes (Figure 6F).

Immune Infiltration Analysis

The immune response, which serves as a vital defense mechanism for the body against external pathogens and maintains homeostasis, plays a complex and central role in the pathogenesis of diseases such as periodontitis, and its dynamic fluctuations are closely linked to disease conditions.^{42,43} Therefore, this study aimed to investigate the interactions between ARGs and immune responses in periodontitis via an immune infiltration analysis.

Through the application of the CIBERSORT algorithm, we quantitatively assessed and subsequently visualized the percentage of DIICs within gingival tissue samples sourced from the GSE16134 (Figure 7A). Clustering heatmaps were generated to illustrate the differences in the percentage of DIICs between the periodontitis gingival group and the healthy gingival group sourced from the GSE16134 dataset (Figure 7B). Furthermore, the correlation heatmap in Figure 7C depicts the relationships among DIICs. Linear regression analysis revealed a noteworthy correlation between hub genes with DIICs in periodontitis (Figure 7D).

In comparison to healthy controls, our analysis revealed a notable rise in the proportions of plasma cells, gamma delta T cells ($\gamma\delta$ T cells), activated memory CD4⁺T cells, and neutrophils were significantly increased in periodontitis tissues. Conversely, periodontitis tissues exhibited a pronounced decline in the proportions of follicular helper T cells (Tfh), resting dendritic cells, CD8⁺T cells, macrophages M1, and resting mast cells (Figure 7E). Therefore, our results suggest that these autophagy hub genes not only play a key regulatory role in autophagy but also modulate immune cell infiltration.

Predicting Potential Autophagy-Regulating Drugs

The prediction of targeted drugs based on protein-drug interactions may provide direction for the treatment of periodontitis. We performed a predictive drug target analysis using the DSigDB database to identify the top 10 autophagy-regulating drugs associated with key genes, sorted by FDR value. These drugs are deemed particularly promising targets for the treatment of periodontitis through autophagy mediation (Figure 8A). The findings indicated that curcumin (Figure 8B) exhibited the strongest drug-target correlation, followed by 27-hydroxycholesterol (Figure 8C) and Trolox (Figure 8D). A comprehensive depiction of the three aforementioned drugs is presented in Figure 8e. These drugs could potentially play a protective role in periodontitis through autophagy mediation, yet more in-depth studies are needed to verify this.

In this study, we used bioinformatics analysis and preliminary in vitro experimental validation to reveal the prevalence of autophagy dysregulation in gingival tissue from patients with periodontitis and successfully screened nine key autophagy-related genes. Further studies showed that changes in the expression of these genes were closely associated with changes in immune cell infiltration. On this basis, we screened drugs, such as curcumin, that can act on these key genes. These findings suggest that dysregulation of autophagy plays an important role in the pathogenesis of periodontitis and provides potential targets and drug candidates for the development of novel therapeutic strategies against periodontitis in the future.

Discussion

Periodontitis, a chronic inflammatory disease of the oral cavity caused by plaque microorganisms, is the primary etiological factor contributing to tooth loss and chewing dysfunction in adults. This condition arises from the prolonged destruction of periodontal supporting tissues.^{44,45} As periodontitis progresses, patients may encounter a significant decline in their quality of life due to associated comorbidities such as dyspepsia and facial aging.⁴⁶ The exact etiology and pathogenesis of periodontitis remain incompletely understood. Previous research suggests that periodontitis may be linked to various pathogenic factors including immunity, inflammation, and environment.⁴⁷ Recently, autophagy in periodontitis pathogenesis has garnered significant attention, with multiple studies emphasizing its importance. Nevertheless, the exact molecular mechanisms remain elusive, highlighting the need for more intensive research to elucidate them.²³

The results from GSEA in this study highlighted significant dysregulation of ARGs in periodontitis gingival tissues, suggesting a crucial role for autophagy in the pathogenesis of periodontitis.^{23,27} Furthermore, experimental investigations using an LPS-induced periodontitis cell model revealed that LPS, a key bacterial component in periodontitis, can induce

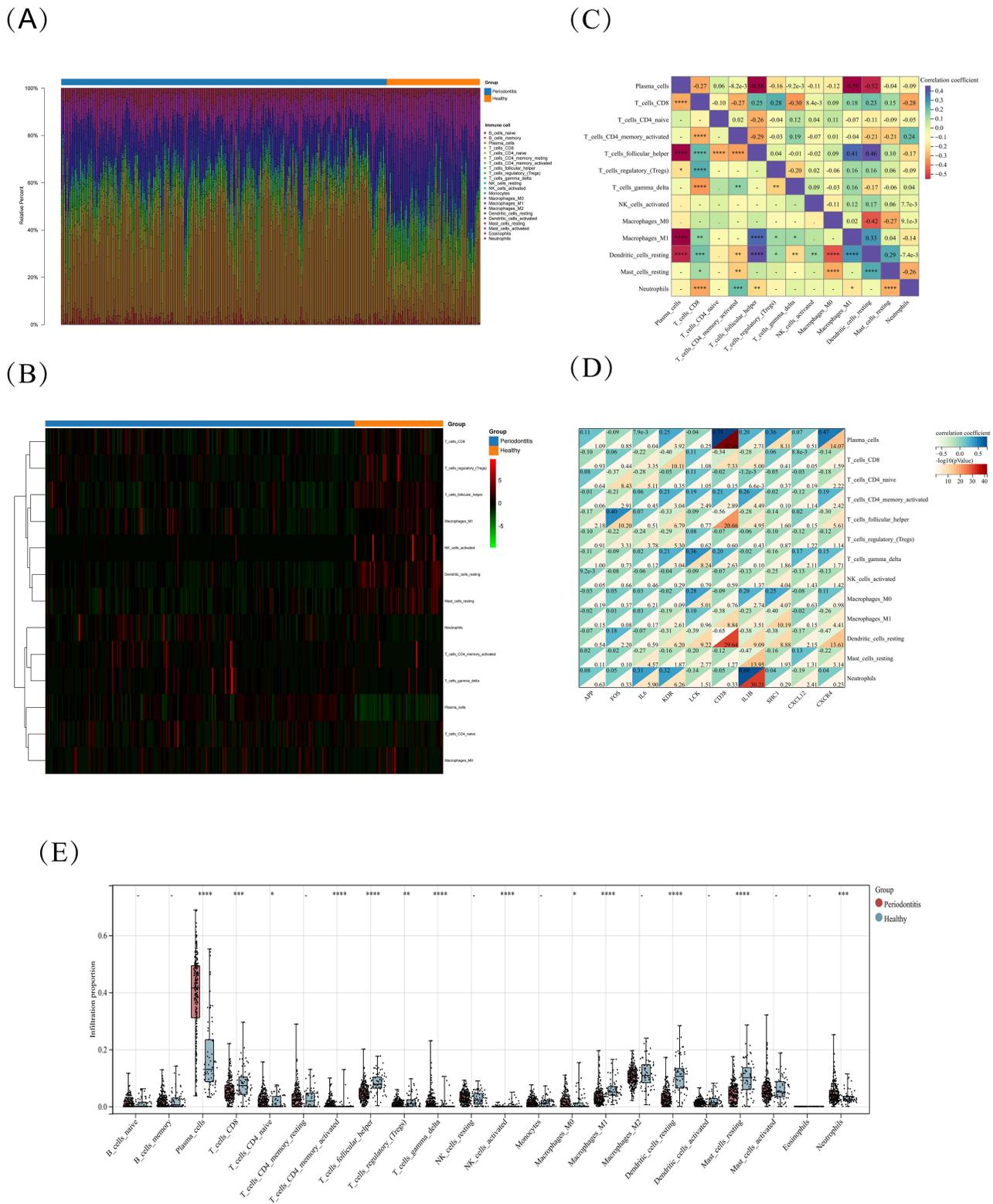
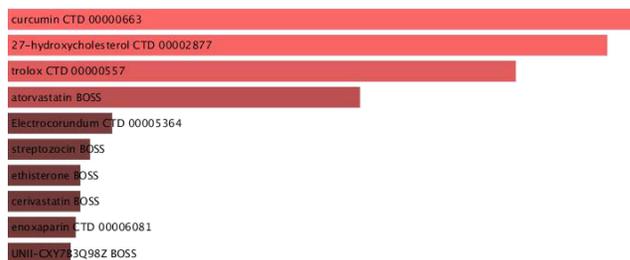
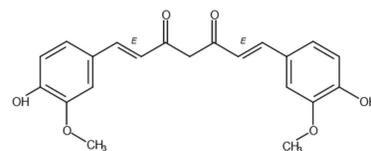


Figure 7 Immune infiltration analysis. In the investigation utilizing the GSE16134 dataset, a notable distinction in the proportion of differentially infiltrating immune cells (DIICs) was identified between gingival tissue samples from healthy controls and individuals with periodontitis. Furthermore, a significant correlation was observed between hub genes and DIICs. **(A)** Evaluation of the percentage composition of DIICs in gingival tissues derived from GSE16134 dataset; **(B)** The clustering heatmaps displaying the differences in the percentage of DIICs between the periodontitis and healthy gingival tissues; **(C)** The correlation heatmap illustrating the correlations among DIICs **(D)** Linear regression analysis revealing a significant correlation between hub genes and DIICs; **(E)** A comparative analysis of DIICs between periodontitis and healthy gingiva tissues in the GSE16134 dataset. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

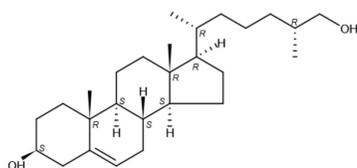
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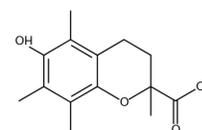
(B)



(C)



(D)



(E)

Predicted drug	Target genes	Odds Ratio	Combined Score	P.Val
Curcumin CTD 0000663	APP;IL6;CXCL12;IL1B;KDR;CXCR4;FOS	87.19321817	1810.263306	0.000000000962
27-hydroxycholesterol CTD 00002877	APP;IL6;CXCL12;IL1B	475.2857143	9793.382912	0.00000000113
Trolox CTD 00000557	IL6;IL1B;KDR;FOS	415.7916667	8362.687649	0.00000000184

Figure 8 Prediction of potential autophagy-regulating drugs. Utilizing the DSigDB database, drugs with autophagy-regulating functions related to key genes were predicted. Among the identified drugs are: curcumin, 27-hydroxycholesterol, and trolox. (A) The top 10 autophagy-regulating drugs associated with key genes according to FDR values sorting. The results showed that curcumin (B) had the highest drug-target correlation, followed by 27-hydroxycholesterol (C) and Trolox (D). A detailed description of the three aforementioned drugs is presented in (E).

autophagic responses in HGFs, leading to heightened autophagy levels. Inhibition of autophagy with 3-MA reduced cytotoxicity and inflammatory responses triggered by LPS, indicating a potential pathological implication of autophagy in periodontitis development.^{23,24} Additionally, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses unveiled that differentially expressed autophagy-related genes (DEARGs) are enriched in processes associated to lymphocyte differentiation, T-cell activation and differentiation, and autophagy. This suggests that dysregulated autophagy might impact immune cell functions, potentially leading to an imbalance in immune responses.²² Such imbalance could manifest as an exaggerated inflammatory response or immunosuppression, exacerbating the damage to periodontal tissues.

We performed a comprehensive bioinformatics analysis using transcriptomic data from a substantial number of human samples, which was further validated through *in vitro* experiments to explore the contribution of autophagy to periodontitis progression. The GSEA results revealed widespread abnormal expression of ARGs in periodontitis gingival tissues, suggesting that autophagy may represent a potential mechanistic pathway in the pathogenesis of periodontitis.^{23,27} Furthermore, GO enrichment analysis was employed to delve deeper into the BP, CC localization, and MF of autophagy-related genes in periodontitis. Accumulating evidence from studies underscores the participation of autophagy in the regulation of T-cell activation and differentiation,⁴⁸ cell adhesion,⁴⁹ and neutrophil homeostasis.⁵⁰ Conversely, T-cell activation and differentiation, as well as cell adhesion can also impact autophagy regulation.^{51,52} The autophagy process within mesenchymal stem cells may regulate the migration and differentiation of CD4⁺ T cell cells via the modulation of CXCL8 and TGF- β 1 signaling pathways.⁴⁸ The differentiation of Th1/Th2 regulated by IRF2-INPP4B promotes autophagy while diminishing apoptosis.⁵² It has been demonstrated that autophagy exerts a modulating influence on the processes of macrophage adhesion and migration in diabetic nephropathy.⁴⁹ Cell adhesion can hinder autophagy through Src/FAK-mediated phosphorylation and AMPK reduction.⁵¹ Our research reveals a significant association between autophagy and protein serine/threonine kinase activity, cytokines, growth factors, heat shock proteins, and misfolded proteins.

Additionally, previous studies have indicated a link between their interactions and various diseases.^{53–57} Indeed, serine/threonine kinases like AMPK, mTOR, and ULK1 play crucial roles in autophagy regulation.^{58–61} This mechanism holds the potential to serve as a pathologic pathway in periodontitis, warranting further investigation. Studies have shown that the inhibition of the Akt signaling pathway can effectively block the positive regulation of mTOR activation by *Porphyromonas gingivalis* (*P. gingivalis*). This disruption results in the downregulation of mTOR and its downstream target ULK1 phosphorylation, ultimately leading to the activation of ULK1 and the initiation of autophagy.⁶² Furthermore, in a high-glucose environment, metformin was found to effectively attenuate periodontal tissue damage in db/db mice. This beneficial effect was attributed to the activation of the AMPK/SIRT1/autophagy signaling pathway mechanism.⁶³ Moreover, the chemokine signaling pathway can impact the disease progression by modulating autophagy,^{64,65} emphasizing the importance of elucidating its regulatory mechanism in periodontitis. Therefore, it is essential to concentrate on unraveling the interactions between these molecules and pathways to comprehensively understand the molecular biological mechanisms governing autophagy regulation in periodontitis.

The construction and subsequent analysis of the PPI network enabled the identification of ten hub autophagy genes associated with periodontitis. Furthermore, our *in vitro* experimentation corroborated the findings of dysregulated autophagic activity in periodontitis and identified nine key genes (APP, KDR, IL1B, CXCL12, CXCR4, IL6, FOS, LCK, SHC1) from the ten hub genes.

Although autophagy-associated genes have been recognized as crucial regulators of pathological mechanisms in numerous diseases, their specific role in modulating autophagy within the context of periodontitis has been seldom reported. Current research on periodontitis indicates that autophagy may influence the production of inflammatory factors, including IL-6 and IL-1b.^{23,66,67} Nevertheless, the exact molecular mechanism governing the interaction of IL-6 and IL-1b with autophagy remains unclear, highlighting an intriguing avenue for further exploration. Bacterial virulence factors such as LPS and leukotoxin have been shown to attack oral epithelial cells, triggering the release of pro-inflammatory factors interleukin-1 alpha (IL-1 α) and interleukin-1 beta (IL-1 β), which play a crucial role in periodontal destruction.⁶⁸ Autophagy acts to eliminate pathogens and inhibit the production of inflammatory factors, thereby mitigating inflammatory responses.⁶⁹ It is conceivable that IL1 β may intensify inflammation in periodontal tissues by influencing the activity of the autophagy pathway. Previous studies have demonstrated that APP expression levels are significantly higher in the gingival tissues of individuals with periodontitis compared to healthy controls.⁷⁰ It is established that autophagy processes regulate the intracellular translocation and processing of APP.⁷¹ We hypothesize that aberrant autophagy could impact the transport and processing of APP during the pathological process of periodontitis, resulting in the accumulation of aberrant proteins in periodontal tissue cells and the exacerbation of inflammation and damage. Therefore, monitoring the expression levels of APP and autophagy-related marker genes could offer valuable insights into assessing the conditions and prognosis of periodontitis. Furthermore, periodontal lesions have been associated with pathological angiogenesis, with the VEGF/VEGFR-2 (KDR) axis identified as a potential factor

influencing the pathological progression of periodontal disease and angiogenesis.⁷² Autophagy also plays a regulatory role in the formation of pathological blood vessels.⁷³ Therefore, the interaction between KDR and autophagy may represent a promising target for anti-angiogenic therapy in periodontitis. Furthermore, the interaction between CXCL12 and its receptor CXCR4 has been associated with immune cell migration in periodontitis.⁷⁴ Autophagy contributes to the immune response within periodontal tissues, influencing the activity and function of immune cells.²² Therefore, the interplay between the CXCL12/CXCR4 axis and autophagy could represent a potential target for immunomodulatory therapy in periodontitis.

This study is the first to reveal an association between CXCL12, KDR, APP, LCK, and SHC1 with the autophagy pathway in periodontitis, relationships that have not been previously documented. This discovery potentially sets the stage for further investigations into the intricate molecular mechanisms underlying periodontitis. Furthermore, while IL1 β and IL6 have been implicated in autophagy in periodontitis, these associations lacked substantiation from high-quality evidence obtained from clinical samples. Our study provides the initial conclusive evidence at the transcriptomic level of the correlation of these genes with autophagy by analyzing a substantial number of clinical samples. Through in-depth investigation of the functions of these autophagy-related genes and their mechanisms of action in periodontitis, there is potential for the development of novel therapeutic strategies aimed at alleviating inflammatory responses and tissue damage, thereby improving patients' oral health status.

The development of periodontitis is characterized by intricate immune and inflammatory responses, with immune dysregulation playing a critical role in its pathogenesis.⁷⁵ The interplay between autophagy and immunity and inflammation is notably complex, as autophagy can modulate immune and inflammatory responses, while immune and inflammatory signals can reciprocally influence autophagy induction and inhibition.⁷⁶ Therefore, the interplay between autophagy and immune cell infiltration in periodontitis warrants attention and further investigations. Our current analysis reveals elevated proportions of plasma cells, T cells gamma delta, Neutrophils, and activated memory CD4⁺ T cells in periodontitis groups compared to that of healthy controls, whereas the percentages of Tfh cells, CD8⁺ T cells, resting mast cells, macrophages M1, and resting dendritic cells were decreased. Some of these findings align with previous studies. In comparison to healthy human gingiva tissues, inflamed gingival tissues from individuals with periodontitis exhibited a significant elevation in the number of CD4⁺ T cells, B cells, and plasma cells.⁷⁷ Additionally, a separate study reported a notable elevation in neutrophil infiltration levels within periodontitis gingival tissues.⁷⁸

Autophagy, a pivotal regulatory mechanism of cellular metabolism, indeed shows pervasive dysregulation in the gingival tissues during periodontitis. Our research findings indicate an increased proportion of activated memory CD4⁺ T cells, plasma cells, and neutrophils in periodontitis — cells closely associated with autophagy. Autophagy exerts a profound influence on the biological functions of CD4⁺ T cells, impacting their proliferation, differentiation, metabolism, and survival, underscoring its crucial role in the regulation of autophagy of periodontal inflammation progression.⁷⁹ Neutrophils play a pivotal role in defending against pathogen invasion via autophagy mechanisms, emphasizing the significance of autophagy in immune defense.²² Furthermore, plasma cells, as specialized antibody-secreting cells, require autophagy regulation during their differentiation process. Autophagy is involved in maintaining endoplasmic reticulum homeostasis and modulating the equilibrium between plasma cell differentiation and antibody production, indicating its essential role in plasma cell function and adaptive immune responses.⁸⁰ In summary, autophagy is intricately intertwined with the functions of CD4⁺ T cells, plasma cells, and neutrophils, collectively governing the immunopathological processes of periodontitis.

Conversely, the infiltration ratio of mast cells, dendritic, and macrophages was decreased in inflamed gingival tissues.⁸¹ Our study, utilizing transcriptome analyses, demonstrated a notable reduction in M1 macrophages in periodontitis. In contrast, a previous investigation reported a significant increase in M1 macrophages within the periodontitis microenvironment in human gingival tissues.⁸² These conflicting findings underscore that the pathogenesis of periodontitis encompasses more than just a spectrum of pathogenic microorganisms and is intricately intertwined with complex immune response mechanisms. As such, as the disease progresses, the quantity and functionality of macrophages are likely subjected to dynamic shifts and alterations.⁸³ *Porphyromonas gingivalis* (*P. gingivalis*) has been found to regulate apoptotic and/or autophagic processes within dendritic cells (DCs), contributing to their survival mechanisms and disrupting immune homeostasis.⁶² Therefore, it is suggested that the restoration of immune homeostasis in patients

with periodontitis should involve boosting antibacterial autophagy and apoptosis. This could help prevent peripheral inflammation and augment bacterial clearance.⁶²

Our investigation revealed a significant correlation between key DEARGs and infiltrating immune cells in periodontitis. It is recognized that certain immune cells may possess dual pro-inflammatory and anti-inflammatory functions. Moreover, as periodontitis advances, the composition of the immune infiltration undergoes dynamic changes. Therefore, a comprehensive study of the close and complex relationship between immune infiltration and autophagy-related genes in periodontitis, as well as the specific regulatory mechanisms, is important for the exploration of novel therapeutic strategies for periodontitis.

In light of the pivotal roles played by key genes in periodontitis and their associated mechanisms, numerous researchers are actively investigating effective therapeutic interventions. In this study, we proposed Curcumin as a potential protective agent against periodontitis by modulating autophagy. Curcumin, a natural compound derived from the rhizomes of plants within the Zingiberaceae and Araceae families,⁸⁴ exhibits diverse biological functions, such as anti-tumor, anti-inflammatory, and analgesic properties.⁸⁵ Previous studies have demonstrated that Curcumin can effectively alleviate and treat periodontitis through various pathways. It has been shown that Curcumin mitigates periodontal damage by inhibiting ferroptosis.⁸⁶ Furthermore, curcumin has been demonstrated to have a suppressing impact on the expression of key virulence genes in *Porphyromonas gingivalis*, resulting in a reduction in its virulence and an improvement in periodontitis.⁸⁷ Notably, Curcumin's therapeutic potential extends to diverse conditions such as tumors, inflammation, and heart disease through the regulation of autophagy.^{88–90}

Previous studies have demonstrated that curcumin possesses the ability to inhibit the growth of periodontal pathogens and their biofilm formation, and can alleviate inflammatory responses in periodontal tissues by inhibiting the activation of the MAPK (mitogen-activated protein kinase) inflammatory signaling pathway.⁹¹ Recent research suggests that curcumin exerts its protective effects by modulating autophagy processes through the regulation of signaling pathways such as AMPK, MAPK, and mTOR.^{92,93} In this study, through GO enrichment analysis, we identified a notable association between DEARGs and protein serine/threonine kinase activity. Based on this finding, we propose a hypothesis that curcumin may influence autophagy processes by regulating signaling pathways such as MAPK, AMPK, and mTOR, which are related to protein serine/threonine kinase activity, thereby potentially alleviating inflammation in periodontal tissues. Nevertheless, this hypothesis necessitates further in-depth research for validation. With its ability to regulate autophagy, curcumin presents itself as a promising and innovative therapeutic strategy for addressing periodontitis.

As a transcriptomic analysis of a limited number of human samples, accompanied by preliminary *in vitro* experimental validation, our study is subject to certain limitations. Firstly, the limitations in the source of transcriptomic samples and sample size may affect the depth of analysis of specific disease stages and complex clinical phenotypes, which in turn reduces the comprehensiveness and accuracy of the findings. It is thought that larger multicentre clinical studies will provide more comprehensive conclusions. Secondly, the environment of *in vitro* experiments is unable to fully replicate the complex environment present in animals or humans. Consequently, *in vitro* experiments can only provide initial validation of bioinformatics findings. Further, *in vivo* experiments and studies with human samples can provide a higher level of evidence for the study, which is the key to further clarifying and translating our findings in the future. It is also important to acknowledge that the various algorithms employed in the bioinformatics analyses of this study may exhibit certain systematic biases. For instance, the use of the CIBERSORT algorithm to determine the proportion of immune cells in human gingival tissues and the prediction of potential therapeutic drugs based on drug-protein relationships are potential sources of bias that, while currently deemed acceptable in the majority of studies, should not be overlooked. Consequently, further in-depth studies are imperative to elucidate the findings.

Conclusion

The present study revealed a general dysregulation of autophagy in periodontitis and identified nine key genes for autophagy regulation (APP, KDR, IL1B, CXCL12, CXCR4, IL6, FOS, LCK, and SHC1) through bioinformatics analysis and *in vitro* experimental validation. Furthermore, our study identified a correlation between these genes and altered immune cell infiltration in periodontitis. Additionally, we predicted potential therapeutic agents, including curcumin, 27-hydroxycholesterol, and Trolox, which exhibit promise in the treatment of periodontitis by mediating autophagy. Our

study provides evidence and novel perspectives to unravel the pathological mechanisms of autophagy regulation in the pathogenesis of periodontitis and to explore new strategies for periodontitis diagnosis and treatment. The conclusions drawn from this study were based on biological information analysis conducted on a relatively limited number of transcriptomes of human gingival tissues, supported by initial in vitro experiments. It is acknowledged that there are several limitations regarding the generalizability and accuracy of these findings, highlighting the critical need for additional validation and refinement through broader and more comprehensive experimental research in the future.

Abbreviations

DEG, differentially expressed genes; ARG, autophagy-related gene; DEARG, differentially expressed ARG; GSEA, Gene set enrichment analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; DIIC, differentially infiltrating immune cell; LPS, lipopolysaccharide.

Author Contributions

All authors contributed to all aspects of this study, from its conception and experimental design to the collection of data, bioinformatics analysis, and in vitro experimental validation. Furthermore, they were actively involved in the writing, revision, and review of the article, ensuring the accuracy and rigor of the research findings. All authors reached a consensus on the submission of the article and assumed full responsibility for the research work.

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Disclosure

The authors declare no competing interests with regard to the paper.

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