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Mechanism of PI3K-AKT Signaling Pathway Mediating Apoptosis of Periodontal Tissue Cells in Chronic Periodontitis

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Abstract
Chronic periodontitis is one of the most common diseases in stomatology, which is characterized by the chronic inflammatory destruction of periodontal supporting tissues and is the most important cause of tooth loss in adults. The aim of this study is to explore the molecular mechanism of chronic periodontitis in Nanning, Guangxi, China, by using high-throughput sequencing technology combined with bioinformatics analysis. A case-control study was conducted. Periodontal tissue samples were collected from 30 healthy patients and 30 patients with chronic periodontitis in the Department of Stomatology, International Zhuang Medical Hospital Affiliated to Guangxi University of Traditional Chinese Medicine. KEGG enrichment and validation results showed that the PI3K-AKT signaling pathway was inhibited in the periodontal tissues of patients with chronic periodontitis in Nanning, Guangxi Zhuang Autonomous Region, which mediated the increase of Caspase9 expression and activated apoptosis, leading to apoptosis and destruction of periodontal tissues. These results suggest that PI3K-AKT signaling pathway may be a new target for clinical treatment of chronic periodontitis in Nanning, Guangxi Zhuang Autonomous Region.
Keywords: *PI3K-AKT signaling pathway, Chronic periodontitis, Apoptosis*

INTRODUCTION

Periodontitis is a chronic inflammatory disease, and its pathological process is characterized by the dysregulation of inflammatory microenvironment mediated by host immune imbalance. Chronic periodontitis is the most common type of periodontitis, accounting for about 95% of patients with periodontitis. Chronic periodontitis has a high incidence in the world. According to the World Health Organization (WHO) survey in 2022, about 20%-50% of adults worldwide suffer from varying degrees of chronic periodontitis. Data from the Fourth China National Oral Health Survey showed that the prevalence of chronic periodontitis in Chinese adults was 52.8%, 69.3%, and 64.6% in the 35-44, 55-64, and 65-74 age groups, respectively (Jiao, 2021). In rural areas of China, the prevalence of chronic periodontitis was higher, reaching 94.1%, and 53.7% of them were moderate to severe. From a trend observation, the incidence and prevalence of chronic periodontitis in Chinese adults have shown an upward trend from 1990 to 2019, and this trend is expected to continue to increase in the next 25 years (Yu, 2023). Among the rest of the world, studies have shown that about 46% to 48% of US adults suffer from chronic periodontitis. In India, the prevalence of chronic periodontitis exceeded 85% in the general population of West Bengal, Uttar Pradesh, and Assam (Shewale, 2016). Chronic periodontitis is the most important cause of tooth agenesis in adults, which seriously affects the chewing, language and aesthetic function of patients. Combined with its high incidence, the medical cost of treatment and restoration caused by chronic periodontitis exceeds 10 billion yuan every year in China, causing a heavy economic burden to the society. At present, the mainstream research on chronic periodontitis mainly focuses on the key genes or molecular signaling pathways of a specific function, and lacks a comprehensive understanding of the pathogenic mechanism of chronic periodontitis patients. Therefore, in order to

provide an overview of the pathogenesis of chronic periodontitis and develop targeted treatment strategies, this study used transcriptome level high-throughput sequencing technology combined with bioinformatics analysis to explore the molecular biological mechanism of chronic periodontitis in Nanning, Guangxi, China. In a previous experiment, we used transcriptome level high-throughput sequencing technology to perform transcriptome level sequencing of periodontal tissues from tooth extraction wounds of chronic periodontitis patients and periodontally-healthy patients in Nanning, Guangxi Province. After bioinformatics analysis of the results, KEGG enrichment results showed that PI3K-AKT signaling pathway was inhibited in periodontal tissues of patients with chronic periodontitis in Nanning, Guangxi Zhuang Autonomous Region. PI3K-AKT signaling pathway is a classical signaling pathway related to phosphatidylinositol in cells, and it is also a derivative of RTK-mediated signaling pathway. The PI3K-AKT signaling pathway plays an important role in the regulation of signal transduction and can regulate biological processes such as apoptosis. It has been confirmed that a variety of tumors have hyperactivation of the PI3K/Akt signaling pathway, which is of great significance for the survival, growth, motility, angiogenesis and metabolism of tumor cells. Therefore, we suggest that the destruction of periodontal supporting tissues in patients with chronic periodontitis is most likely related to the apoptosis process mediated by PI3K-AKT signaling pathway activation in periodontal tissues. This study further verified the expression of downstream key factors of PI3K/AKT signaling pathway in periodontal tissues of patients with chronic periodontitis in Nanning, Guangxi. In addition, inhibition of PI3K/AKT signaling pathway can be used as a marker for the pathogenesis of chronic periodontitis, which lays a theoretical foundation for the prevention and treatment of chronic periodontitis. It also provides a theoretical basis for future targeted gene therapy for patients with chronic periodontitis in Nanning, Guangxi. The study is the first of its kind in the world.

Research Objectives

To investigate the molecular biological regulation mechanism of key factors in PI3K/Akt signaling pathway mediating the apoptosis of periodontal tissue cells in patients with chronic periodontitis in Nanning, Guangxi.

Scope of the Research

1. Population and sample

The overall population of this study was a group of adults over 18 years of age attending the Department of Stomatology, International Zhuang Medical Hospital Affiliated to Guangxi University of Traditional Chinese Medicine. The study sample was the patients who needed tooth extraction treatment in the Department of Stomatology, International Zhuang Medicine Hospital Affiliated to Guangxi University of Traditional Chinese Medicine from May 2024 to October 2024. Thirty healthy patients with periodontal tissue and 30 patients with chronic periodontitis who needed tooth extraction treatment in the Department of Stomatology, International Zhuang Medicine Hospital Affiliated to Guangxi University of Traditional Chinese Medicine from May 2024 to October 2024 were selected, with a total of 60 samples.

2. Regional scope

The study area was Nanning city, Guangxi Zhuang Autonomous Region, China.

3. Time frame

The time frame of the study was May 1, 2024, to October 31, 2024.

RESEARCH METHODOLOGY

1. Periodontal tissue collection. Thirty patients with healthy periodontal tissue (control group) and 30 patients with chronic periodontitis (chronic periodontitis group) who needed tooth extraction treatment in the Department of Stomatology, International Zhuang Medicine Hospital Affiliated to Guangxi

University of Traditional Chinese Medicine from May 2024 to October 2024 were selected. Informed consent was obtained after tooth extraction examination and exclusion of surgical contraindications. The tooth extraction was completed under local anesthesia, and the periodontal ligament tissue in the tooth extraction socket was collected. The size of the periodontal ligament tissue was about 0.5cm×0.5cm, and the periodontal ligament tissue was preserved according to different specimen preservation methods for the following experiments.

2. High-throughput sequencing at the transcriptome level was performed on the periodontal ligament tissues of the chronic periodontitis group and the control group. Total RNA was extracted from the chronic periodontitis group and the control group by Trizol method. The TruseqTM Small RNA sample prep kit from Illumina was used to construct cDNA libraries. The cDNA library was sequenced using the Illumina Hiseq 4000 sequencing platform, and the results were statistically analyzed.

3. Quantitative Real-Time PCR (qPCR).

Apoptosis and PI3K/AKT signaling pathwayrelated gene expressions were assessed via qPCR. Briefly,cellular RNA was collected using TRIzol (Invitrogen, CA,USA) based on provided directions, and cDNA was prepared using a reverse transcription kit (Invitrogen). A

QuantStudio-5 system (Applied Biosystems, USA) was then utilized for all qPCR amplification using the primers shown in Table 1. Relative gene expression was assessed via the $2-\Delta\Delta CT$ method, with GAPDH being used for normalization.

Table 1: Primer sequences.

Gene	Forward (5' -3')	Reverse (5' -3')
PI3KCA	AAACAGAGCCAAAGGGAAGG	ATACCAGCCACAAAGGCTTC
AKT-1	ATCGCTTCTTTGCCGGTAT	TCTTGGTCAGGTGGTGTGAT
PTEN	CGGAATTCGGATGTCCCGAAAGCAG G	CCGCTCGAGTCAGATGTTGAGCG G
Caspase 9	TGGAACAAATGGACCTGTTGACC	AGGACTCAAATTCTGTTGCCACC
GAPDH	CCACTTTGTGAAGCTCATTTCCT	TCGTCCTCCTCTGGTGCTCT

4. Western-Blot. Briefly, 12% SDS-PAGE was used to separate equal protein samples (30 μg each), which were then transferred to PVDF membranes. Blots were next blocked for 1 h using 5% non-fat milk in TBST, prior to incubation overnight with antibodies specific for PI3KCA, AKT1, p-AKT1, JAK1, Caspase9, and β-actin (all 1:1000 in TBST) at 4 °C. Blots were then washed thrice using TBST (5 min/wash) prior to an additional 1 h room temperature incubation with biotinconjugated secondary antibodies (1:1000 in TBST). A BeyoECL Plus kit was then used to detect all protein samples based on provided directions, with ImageJ (https://imagej.nih.gov/ij/) being used for densitometric analyses. All experiments were repeated a minimum of three times.

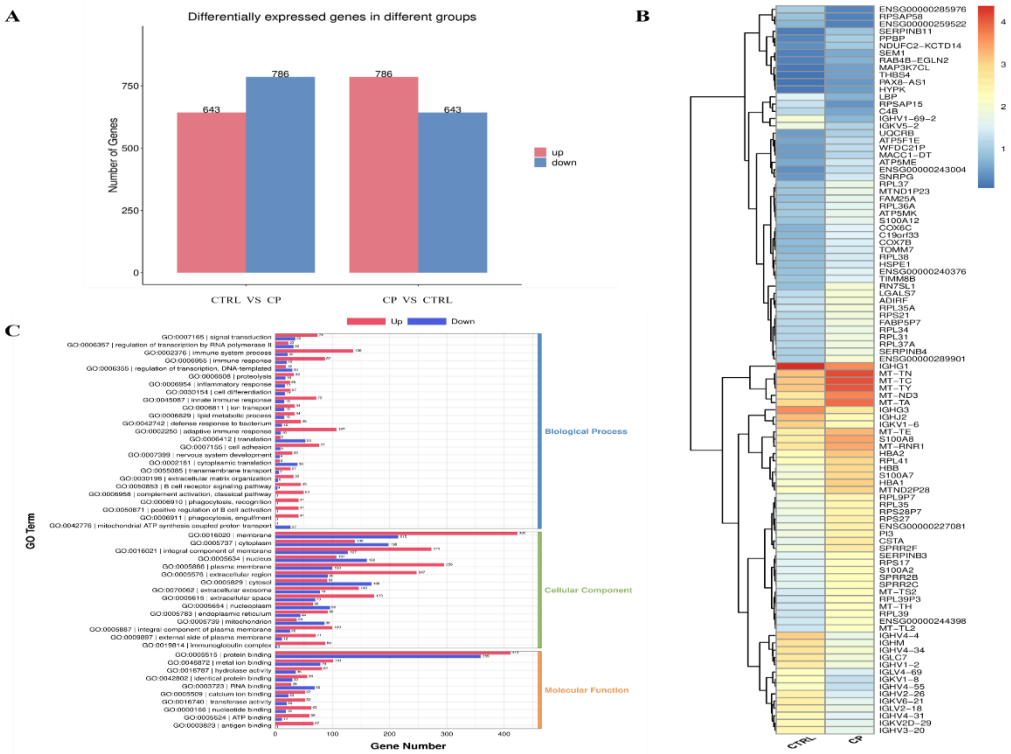
5.Tissue samples that had been fixed were embedded in paraffin and sectioned. Antigens were repaired at high pressure and blocked with 5% BSA at 37℃ for 30 min. Caspase9 antibody (1/200) was incubated in a wet box overnight at 4℃. The cells were incubated with fluorescent secondary antibody cy3 (1/200) for 45 min at 37℃, and DAPI was dropped and incubated in the dark for 3 min. Finally, the slices were observed under a fluorescence microscope and images were collected.

Research Results

1. Differential expression analysis (DEGs) : The transcriptome high-throughput sequencing data of the control group and the chronic periodontitis group were analyzed to screen out the significantly differentially expressed genes. The number of differentially expressed genes up-regulated ($\log_2$FC >= 1 &

q <0.05) and down-regulated (log_2FC <= -1 & q <0.05) in each group was counted, and the statistical bar chart of differentially expressed genes was as follows. Among them, 786 genes were up-regulated in the chronic periodontitis group and 643 genes were down-regulated in the control group (Figure 1 (A)). According to the similarity degree of gene expression profiles of samples from CP group and CTRL group, the differentially expressed genes were analyzed by cluster analysis, and the differentially expressed Top100 genes were displayed by heat map (Figure 1 (B)). The Top25 of the significantly different genes enriched in terms of Biological Process, Cellular Component, and Molecular Function were plotted and displayed (Figure 1 (C)). The results showed that there were a large number of differentially expressed genes between the CP group and the CTRL group, and many different functions were identified by cluster analysis, suggesting that chronic periodontitis may be the result of the joint regulation of multiple factors and biological mechanisms.

Fig.1 Gene expression differences and cluster analysis. A Number of differentially expressed genes



between the CP and CTRL groups, with up-regulated genes in red and down-regulated genes in blue. Heat map of Top100 differentially expressed genes with the smallest B q value, color from blue through white to red indicates expression from low to high. Red indicates highly expressed genes and blue indicates low expressed genes. C GO TERM. The ordinate represents the number of differentially expressed genes enriched to GO entries, the number of up-and down-regulated genes is red and blue, respectively, and the abscissa represents GO entries.

2.KEGG enrichment analysis of differentially expressed genes: the top 20 pathways with the lowest q value of KEGG enrichment analysis were screened to show the distribution of up-regulated and down-regulated differentially expressed genes. KEGG enrichment results showed that 31 genes were up-regulated and 8 genes were down-regulated in PI3K-AKT signaling pathway (Figure 2 (B)). KEGG enrichment bubble map was used to display the Top20 Pathway with the lowest q value, and the results showed that the number of differential genes between CP group and CTRL group was significantly enriched in PI3K-AKT signaling pathway. The PI3K-Akt signaling pathway plays a crucial role in regulating cellular processes such as survival, proliferation, differentiation, and metabolism. This

pathway is initiated when phosphoinositide 3-kinase (PI3K) is activated by extracellular signals such as growth factors or cytokines. Activated PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits and activates Akt (protein kinase B) at the plasma membrane. Akt phosphorylates downstream targets involved in anti-apoptotic signals, cell cycle progression, and immune responses.Therefore, it can be speculated that the destruction of periodontal supporting tissues in patients with chronic periodontitis may be related to the apoptosis process of periodontal supporting tissues mediated by the activation of PI3K-AKT signaling pathway.

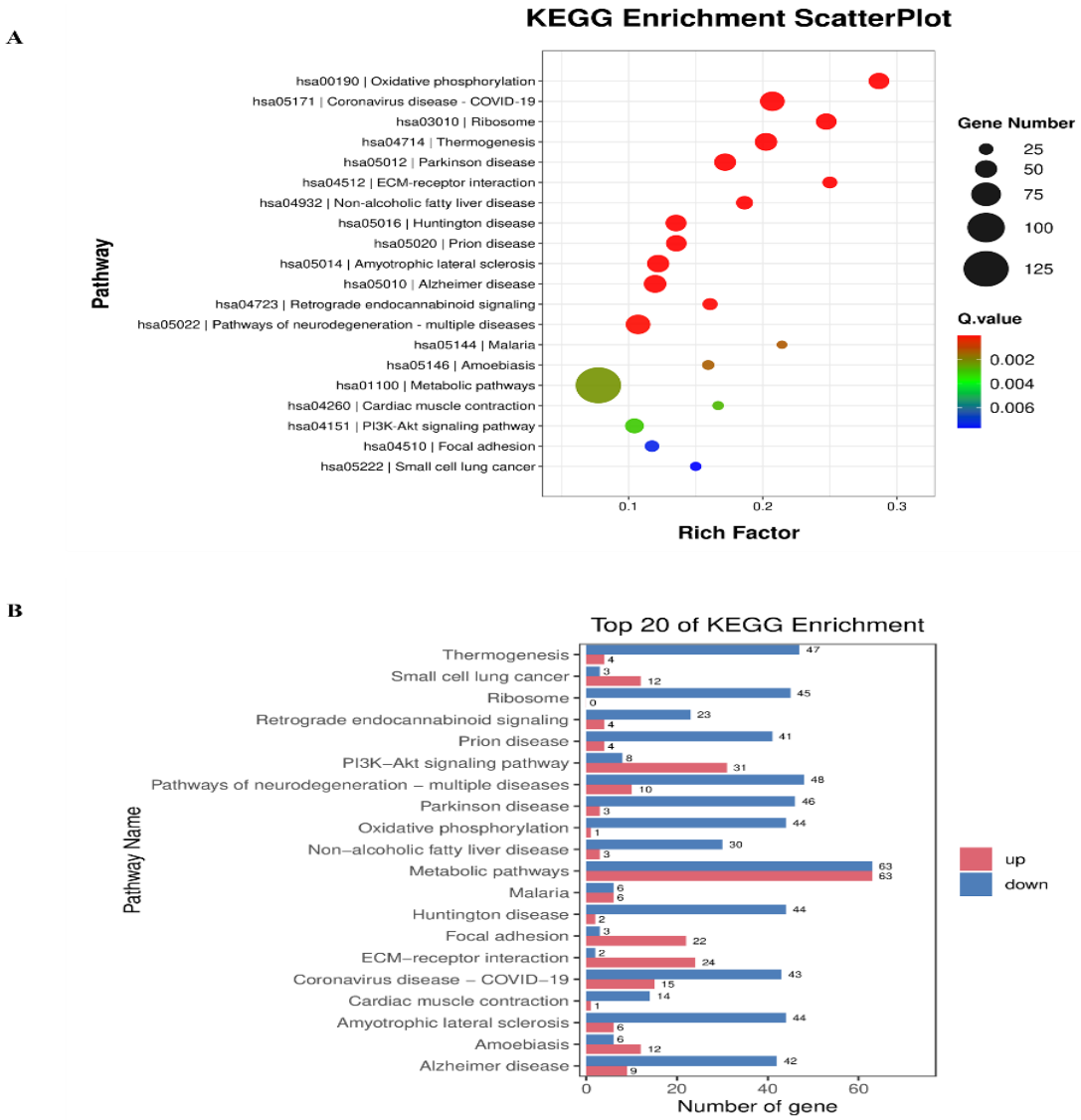


Fig.2 KEGG enrichment analysis. A KEGG Enrichment Scatterplot. B Top20 of KEGG enrichment

3.In order to verify the expression difference of PI3K-AKT signaling pathway shown in KEGG enrichment analysis results between the CP group and the CTRL group, the key genes of PI3K-AKT signaling pathway (PI3KCA, AKT-1, PTEN) and the key downstream factor Caspase9 were screened for qPCR. The results showed that compared with the CTRL group, the relative expression levels of PI3KCA and AKT-1 mRNA in the CP group decreased ($P < 0.05$), and the relative expression levels of PTEN and Caspase9 mRNA increased ($P < 0.05$) (Figure 3 (A)). The amplification curves of each gene are shown below (Figure 3 (B)).

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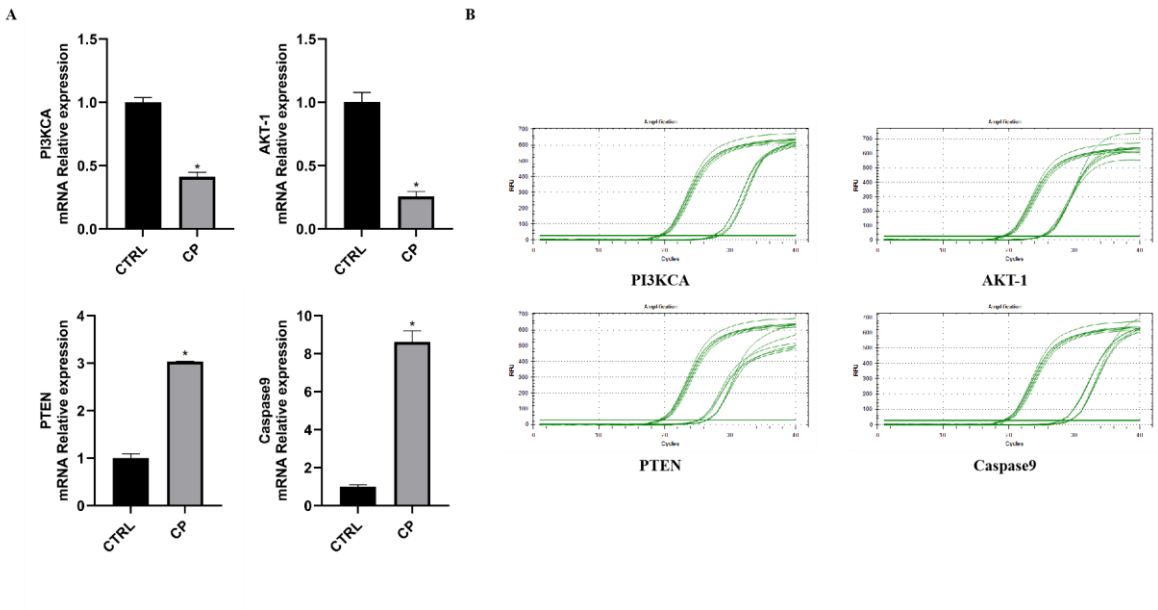

Fig.3 qPCR. A qPCR analysis for the expression of PI3KCA、AKT-1、Jak1、PTEN and Caspase9. B Amplification curve.

4.In order to verify the difference of protein expression of key genes in PI3K-AKT signaling pathway between the CP group and the CTRL group, we detected the protein expression of PI3KCA, AKT-1, p-AKT-1, PTEN and Caspase9 by Western-Blot. The results showed that compared with the CTRL group, the protein relative expression levels of PI3KCA, AKT-1 and p-Akt-1 in the CP group were decreased ($P < 0.05$), and the protein relative expression levels of PTEN and Caspase9 were increased ($P < 0.05$). There was no significant difference in the proportion of p-Akt-1 ($P > 0.05$) (Figure 4).

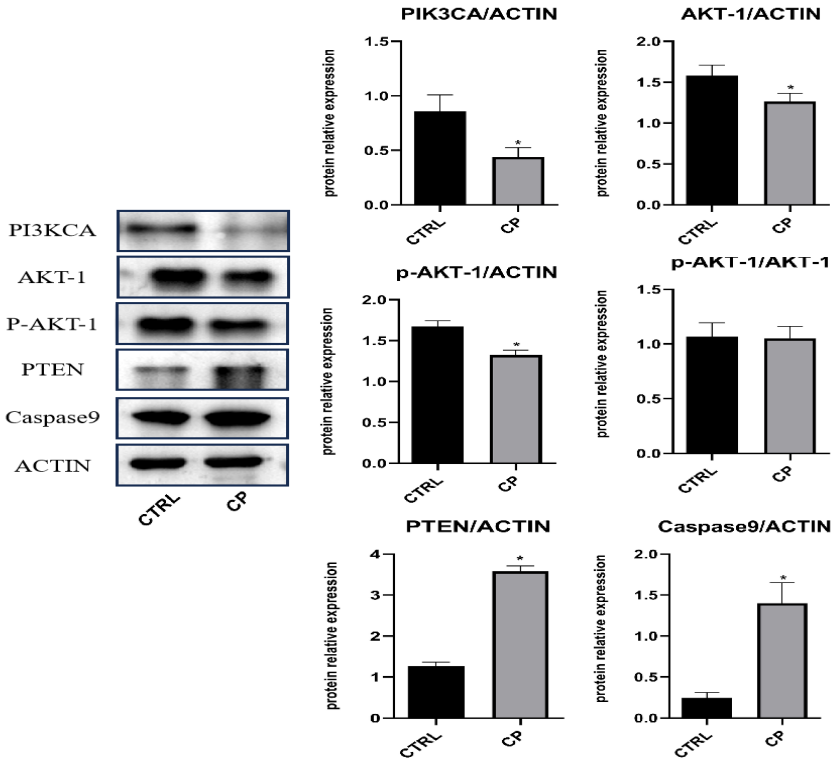

Fig.4 Western-Blot analysis for the expression of PI3KCA、AKT-1、p-AKT-1、PTEN and Caspase9.

5.In order to verify the expression of Caspase9 in chronic periodontitis tissues, the expression and localization of Caspase9 were detected by immunofluorescence staining. The results showed that Caspase9 was highly expressed in the cytoplasm of periodontal tissues in the CP group compared with the CTRL group ($P < 0.05$).

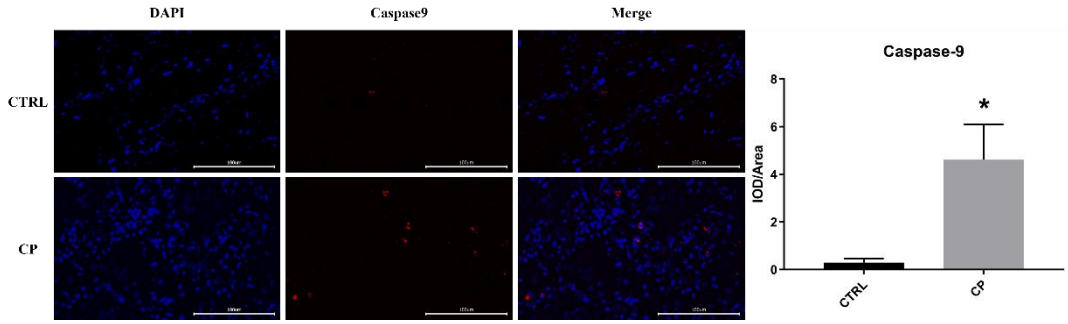

Fig.5 Immunofluorescence staining analysis for the expression of Caspase9.

DISCUSSION

Chronic periodontitis is a chronic inflammatory disease induced by plaque biofilms and characterized by the progressive destruction of periodontal supporting tissues (gingiva, periodontal ligament, and alveolar bone). Recent studies have found that abnormal apoptosis of periodontal tissue cells (such as gingival fibroblasts, periodontal ligament cells, and osteoblasts) is one of the important mechanisms of disease progression. The PI3K-AKT signaling pathway plays an important role in the regulation of cell survival and apoptosis. PI3K/AKT signaling pathway regulates apoptosis mainly by inhibiting apoptotic signals and promoting cell survival signals.

1. Mechanisms of inhibition of apoptosis

NF-kappaB activation: PI3K and AKT inhibit tumor necrosis factor (TNF) -induced apoptosis by activating the NF-kappaB signaling pathway. This activation is both dependent on and independent of TNFR signaling, suggesting that AKT inhibits apoptosis through multiple pathways (Burow, 2000).

2. Negative regulation of JNK pathway: In influenza virus infection, the PI3K/AKT pathway protects cells by inhibiting the JNK pathway to reduce Bax-mediated apoptosis 7. (Lu, 2010)

Regulation of mTOR and autophagy(Fattahi, 2020): in chronic obstructive pulmonary disease (COPD) model, PI3K/AKT/mTOR pathway reduces alveolar epithelial cell apoptosis by promoting autophagy (Zhang, 2020).In this study, high-throughput sequencing and KEGG enrichment results showed differential expression of PI3K-AKT signaling pathway genes, suggesting that periodontal tissue destruction in chronic periodontitis may be related to the inhibition of PI3K-AKT signaling pathway, leading to abnormal apoptosis of periodontal tissue cells.

In this study, we validated the high-throughput sequencing results in periodontal specimens from patients with chronic periodontitis. Firstly, qPCR and Western-Blot results showed that the expression of PI3KCA and AKT-1 was decreased in chronic periodontitis, and Western-Blot results showed that p-AKT-1 expression was decreased. The PIK3CA gene encodes the p110α protein, which is part (subunit) of the PI3K enzyme. The p110α protein is a catalytic subunit responsible for PI3K action, and another PI3K subunit encoded by another gene is responsible for regulating PI3K enzyme activity. The main function of PI3K enzyme is phosphorylation, which triggers a series of intracellular signal transmission through the phosphorylation of other proteins. AKT-1 is a key molecule in cell signaling pathways, which participates in the regulation of cell growth, survival, metabolism and differentiation by phosphorylating different target proteins. AKT-1 plays a role in a variety of cell signaling pathways, including the PI3K/AKT pathway that promotes cell survival and the mTOR pathway that regulates the cell cycle. The

decreased expression of PI3KCA, AKT-1 and p-AKT-1 indicated the inhibition of PI3K-AKT signaling pathway in chronic periodontitis. Meanwhile, qPCR and Western-Blot results showed that the expression of PTEN increased. PTEN is a major negative regulator of the PI3K/AKT signaling pathway(Noorolyai, 2019), degrading PIP3 through its lipid phosphatase activity, thereby inhibiting AKT activation. Inactivation or loss of PTEN is common in a variety of tumor types, leading to excessive activation of PI3K/AKT signaling pathway and promoting tumorigenesis. Studies have shown that PTEN may also participate in cellular regulatory processes through dephosphorylation. The decreased expression of p-AKT-1 may be related to the increased expression of PTEN, which further indicates that PI3K-AKT signaling pathway is inhibited in chronic periodontitis.

Caspase9 is called an "initiating" or "naive" Caspase because it initiates activation in a series of apoptotic signaling pathways and is an important node in apoptotic signaling(Yin, 2006). In the apoptotic pathway, Caspase9 activation usually occurs by the formation of a Caspase9 heterodimer. After the formation of this dimer, Caspase9 is further activated, which initiates further activation of downstream executive caspases, such as Caspase3 and Caspase7, initiating an apoptotic response(Araya, 2021). Caspase9 activation can be mediated by a variety of signaling pathways, the most typical of which is the mitochondrial pathway(Zhang, 2020). In this pathway, cells release cytochrome c in mitochondria due to DNA damage, cell stress or hypoxia. Cytochrome C binds to Apaf-1 (apoptotic protease activating factor-1) and ATP to form a complex that activates Caspase9. The PI3K/AKT signaling pathway affects Caspase9 activation by regulating apoptosis-related proteins. Specifically, inhibition of PI3K/AKT signaling leads to Caspase9 activation, which promotes apoptosis. In multiple cell types, inhibition of PI3K/AKT signaling leads to dephosphorylation of the pro-apoptotic protein BAD, which in turn releases cytochrome c and activates Caspase9, ultimately triggering apoptosis. (Jeong, 2008; Zhao, 2004; Georgakis, 2010).In the present study, Caspase9 expression was significantly increased and abundantly expressed in the cytoplasm of periodontal tissues, suggesting that the development of chronic periodontitis may be related to the inhibition of PI3K/AKT signaling pathway to mediate Caspase9 activation. Therefore, targeting the PI3K/AKT signaling pathway may be a potential means for the clinical treatment of chronic periodontitis. Through the targeted regulation of key genes in PI3K/AKT signaling pathway, promoting PI3K/AKT signaling pathway signaling transduction, reversing the expression of Caspase9 and reducing the apoptosis of periodontal tissue cells, it will provide a new idea for the clinical treatment of periodontal tissue destruction in chronic periodontitis.

Clinical trial number

Not applicable.

Ethical Approval

All experimental procedures were approved by Guangxi University of Chinese Medicine Institutional Review Board.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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