Investigation of Salivary miRNA-155 Levels in Patients with Periodontitis and Plaque-Induced Gingivitis: A Cross-Sectional Study

Periodontitis ve Plak Kaynaklı Diş Eti İltihabı Olan Hastalarda Tükürük miRNA-155 Düzeylerinin Araştırılması: Kesitsel Çalışma

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Citation: Akdeniz T, Nalbantoğlu AM. Barut Z. Investigation of Salivary miRNA-155 Levels in Patients with Periodontitis and Plaque-Induced Gingivitis: A Cross-Sectional Study. *Int Arc Dent Sci.* 2025; 46(2): 113-119.

ABSTRACT

INTRODUCTION: Periodontal diseases are characterized by the progressive inflammation of periodontal tissues, leading to functional impairment and substantial economic burdens. The utilization of biomarkers in early diagnosis and prognosis is a promising approach for predicting disease progression and optimizing treatment strategies. This study aims to evaluate the potential use of miRNA-155 as a biomarker for the early diagnosis of periodontitis and gingivitis.

MATERIAL and METHODS: This study involved seventytwo systemically healthy non-smoker individuals. Based on periodontal characteristics participants were categorized into three equal study groups: periodontal health, gingivitis, and Stage III periodontitis (n = 24). Saliva samples collected from all participants and miRNA expression analysis was performed utilizing the miRCURY LNA SYBR Green PCR Kit. The data were statistically analyzed using SPSS software.

RESULTS: miRNA-155 expression levels demonstrated statistically significant differences between the periodontal healthy, gingivitis and periodontitis groups. A decrease in miRNA-155 expression was observed in the gingivitis group compared to healthy individuals, while a marked increase in miRNA-155 expression levels was detected in the periodontitis group.

CONCLUSION: The findings of this study suggest that miRNA-155 is actively involved in the inflammatory modulation responsible for the development of periodontitis and may potentially serve as a diagnostic biomarker for the disease.

Keywords: *Periodontal diseases, periodontitis, gingivitis, miRNA-155, biomarker*

ÖZ

GİRİŞ: Periodontal hastalıklar, periodontal dokuların ilerleyici inflamasyonu sonucu fonksiyon kaybı ve ekonomik yük oluşturan hastalıklardır. Biyobelirteçlerin erken teşhis ve prognozda kullanımı, hastalığın seyrinin öngörülebilmesi ve tedavi stratejilerinin optimize edilmesi açısından potansiyel bir araç olarak önem arz etmektedir. Çalışmamızın amacı miRNA-155'in periodontitis ve gingivitisin erken tanısında biyobelirteç olarak kullanım potansiyelini değerlendirmektir.

YÖNTEM ve GEREÇLER: Bu çalışmaya sistemik olarak sağlıklı sigara içmeyen yetmişiki gönüllü katıldı. Katılımcılar periodontal karakterlerine göre üç eşit çalışma grubuna ayrıldı: periodontal sağlıklı, gingivitis ve Evre III periodontitis (n = 24). Tüm katılımcılardan toplanan tükürük örneklerinde miRNA-155 seviyeleri miRCURY LNA SYBR Green PCR Kit kullanılarak analiz edildi. Veriler istatistiksel olarak SPSS software ile değerlendirildi.

BULGULAR: miRNA-155 ekspresyon seviyeleri, gruplar arasında istatistiksel olarak anlamlı farklılıklar gösterdi. Sağlıklı bireylere kıyasla gingivitis grubunda miRNA-155 ekspresyonunda azalma gözlemlenirken, periodontitis grubunda bu seviyelerde belirgin bir artış tespit edildi.

TARTIŞMA ve SONUÇ: Bu çalışmanın bulguları, miRNA-155'in periodontitisin gelişiminden sorumlu inflamatuar modülasyonda aktif olarak yer aldığını ve potansiyel olarak hastalık için bir tanı biyobelirteci olarak kullanılabileceğini düşündürmektedir.

Anahtar Kelimeler: *Periodontal hastalıklar, periodontitis, gingivitis, miRNA-155, biyobelirteç*

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Received Date: 25.02.2025
Accepted Date: 24.04.2025

INTRODUCTION

Periodontal diseases (PD) are chronic inflammatory disorders affecting gingiva and supporting tissues. Conditions such as periodontitis and plaque-induced gingivitis are particularly prevalent within populations, posing a significant economic burden on global healthcare systems.[1]

Gingivitis is an inflammatory condition confined to the soft tissues above the marginal bone, characterized by signs such as bleeding on probing, swelling, and redness in the interdental papillae. Weakened host response, along with risk factors, increased levels of inflammatory mediators, and pathogenic bacteria, can facilitate the progression of gingivitis to periodontitis.[2]

In periodontitis, the inflammation spreads to the deeper periodontal tissues, resulting in alveolar bone resorption and loss of connective tissue attachment. The pathogenesis of the disease is intricately linked to the host immune response, wherein nuclear factor-kappa B (NF-Kb) activation plays a pivotal role in facilitating bone loss by modulating osteoblastic activity.[3]

MicroRNAs (miRNAs) are nearly 19-22 nucleotides long, single-stranded RNAs, and have a vital function in regulating gene expression after transcription. They exhibit regulatory effects on both the innate and acquired immune systems.[4] Secreted miRNAs are identifiable in a range of body fluids, including spinal fluid and blood serum, along with gingival crevicular fluid (GCF) and saliva.[5] Circulating miRNAs, which can remain stable in human biofluids, are regarded as promising indicators for diagnosing and predicting the course of diseases. Additionally, miRNA-based therapies are considered to hold significant potential for the treatment of various diseases.[6]

MiRNAs have been shown to exhibit distinct profiles in diseased tissues associated with various pathological conditions, including PD. These differential miRNA profiles offer significant diagnostic potential as biomarkers or prognostic indicators.[7,8]

MiRNAs regulate gene expression by facilitating mRNA degradation or preventing its translation thereby controlling the differentiation, and survival of osteoblasts. In this capacity, they play a critical role in osteogenesis, spanning processes from the formation of bones during embryonic stages to the preservation of adult bone tissue.[9,10] Osteoclastogenesis, is also regulated by miRNAs through the direct modulation of osteoclast activity, signaling cascades, or inhibitory feedback mechanisms.[11]

MiRNA-155 can be defined as a multifunctional microRNA derived from the BIC gene located on chromosome 21, which is crucial in regulating immune responses or inflammation.[12] It is present in hematopoietic progenitor and stem cells, in hematopoietic cells, such as B cells, T cells, monocytes, or granulocytes.[13] This miRNA regulates the $CD4^+$ T cells differentiation, particularly influencing the development of helper T cells and regulatory T cells (Tregs). Additionally, it is crucial for the proper differentiation of B cells and the production of antibodies.[14] MiRNA-155, which participates in multiple biological reactions, including hematopoiesis, inflammation, or immunity, plays a pro-inflammatory role by deactivating anti-inflammatory cytokines.[15] In vitro studies have demonstrated that activated NF-κB enhances the expression of miRNA-155, subsequently activating downstream the genes related to pro-inflammatory cytokine.[11]

MiRNA-155 has been demonstrated to promote macrophage pyroptosis during the early infection phase of P. gingivalis, a bacterium frequently associated with PD. Its inhibition has been demonstrated to reduce pyroptosis rates in P. gingivalis-stimulated macrophages, thereby enhancing their phagocytic ability to eliminate the bacteria.[16]

MiRNA-155 expression increases rapidly in response to infections and injuries, triggered by inflammatory stimuli and hypoxia. In contrast, anti-inflammatory cytokines and negative regulators suppress miRNA-155 expression, thereby terminating the immune response.[8]

It has also been identified to display both pro-inflammatory and anti-inflammatory characteristics. Its dysregulated expression is associated with chronic inflammatory diseases, highlighting its role as a key modulator of inflammatory mechanisms.[17] Zheng et al.[10] demonstrated that miRNA-155 negatively impacts bone regeneration and reduces bone mass. This finding underscores the potential therapeutic significance of miRNA-155 inhibitors in promoting bone regeneration under inflammatory conditions.[10] Additionally, miRNA-155 has been shown to function as a negative regulator by suppressing osteogenic differentiation triggered by bone morphogenetic protein 9 located in mesenchymal cells.[18]

Numerous studies suggest that miRNAs hold significant potential as effective biomarkers and therapeutic targets in the identification of diseases, prognosis, and trerapy of various diseases.[5, 9, 12, 15] MiRNA-155, the focus of this study, is recognized for its pivotal role in inflammatory and immune response processes. Our study aims to investigate the potential of miRNA-155 as an indicator for early diagnosis of pathological conditions such as periodontitis and plaque-induced gingivitis, as well as its viability as a treatment target.

MATERIAL AND METHODS

This research adhered to the Helsinki Declaration and received approval from the S.D.U Medical Ethics Board

(05.11.2024. No: 38). Written and Verbal consent was acquired from all participants before the procedures. A statistical power analysis was carried out via GPower version 3.1 to determine the required sample size (Heinrich-Heine-University, Germany). With an effect size of 0.25 and a power level of 90%, the total sample size was calculated. The study was performed with a total of 72 volunteers, consisting of 24 participants in each group.

A total of 72 systemically healthy, non-smoking individuals were included. Eligibility criteria mandated that participants should not have systemic diseases, be on current medications, or have crowns, veneers, or restorations, and must possess at least 20 teeth in their mouths. Exclusion factors encompassed the presence of systemic diseases, active medication usage, pregnancy, menstrual periods, periodontal therapy within the last six months, or the use of antibiotics or anti-inflammatory medications, either locally or systemically, in the previous two weeks.

Participants with PD were categorized into three groups according to their periodontal status: periodontitis (Group P), gingivitis (Group G) and healthy periodontium (Group H) with 24 individuals in each group. The definitions of gingivitis and periodontitis were established according to the revised 2017 Classification of Periodontal and Peri-Implant Diseases and Conditions by the World Workshop on Periodontology.

Participants in Group H exhibited a periodontium in a healthy state, characterized by the absence of clinical attachment loss (CAL), probing depths (PD) of 3 mm or less, and bleeding on probing (BOP) scores lower than 10% at all examined sites. Group G consisted of individuals diagnosed with gingivitis, showing no CAL and alveolar bone loss on radiographic images, PD less than 3 mm, and BOP levels of 10% or higher at all sites. Group P included individuals diagnosed with Stage III, Grade B periodontitis, with at least two non-adjacent sites exhibiting PDs of 6 mm or greater and CAL of 5 mm or greater, along with radiographic analysis demonstrated a bone loss ranging from 15–35% or extending to the middle third of the root or beyond.

Panoramic radiographs were utilized to assess the loss of alveolar bone. Clinical periodontal parameters, including CAL, PD, and plaque index (PI), were evaluated at six sites per tooth using a periodontal probe. The PI was assessed following the Silness and Löe plaque index criteria.

Unstimulated saliva samples were collected for the study. Participants were warned not to eat, drink or chew gum at least one hour before collecting the samples. Saliva samples were obtained between 9:00 AM and 11:00 AM under similar room conditions. Unstimulated saliva was collected using plastic containers and then transferred to centrifuge tubes via sterile syringes.

To enhance the accuracy of analysis and reduce turbidity, the saliva samples were vortexed at 23 °C for 10 minutes. The supernatants were carefully obtained and stored at -80°C until using miRNA analysis.

After thawing, the saliva samples were centrifuged +4ºC for 20 minutes at 11.000 g and miRNAs were extracted from the supernatant utilizing the miRNeasy Serum/Plasma Kit (Cat. No./ID: 217184, Qiagen, Germany) following the manufacturer's guidelines. The NanoDrop 2000 spectrophotometer was employed to determine the purity and concentration of the extracted miRNAs (Thermo Scientific, Waltham, MA, USA).

cDNA synthesis was carried out using the miRCURY LNA RT Kit (Cat. No./ID: 339340, Qiagen, Germany). The isolated miRNA samples underwent reverse transcription during the cDNA synthesis process. The concentration of transcribed miRNAs was quantified using the Qubit 3.0 Fluorometer (Thermo Scientific) following the standard protocol of the Qubit miRNA Assay Kit. After concentration measurements, appropriate dilutions were prepared as needed.

Following the quantification of sample concentrations, the expression levels of microRNA-155 (Cat. No./ID: 205986-1, Qiagen, Germany) were determined using the miRCURY LNA SYBR Green PCR Kit (Cat. No./ID: 339346-1, Qiagen, USA) on a Rotor-Gene Q real-time PCR system (Rotor-Gene Q, Qiagen). The housekeeping gene RNU6 (lot: 20800469-1, Qiagen Germany) was utilized as an internal control for normalization.

Statistical analyses were performed via SPSS software, version 26.0. The normality was assessed using the Kolmogorov-Smirnov test, and the homogeneity was evaluated with Levene's test. Differences in PI, gingival index, and PD among the study groups were analyzed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey tests. The levels of miRNA-155 among the study groups were analyzed using the Kruskal-Wallis test. Statistical significance was arranged at $p < 0.05$ with a 95% confidence interval.

RESULTS

In this research, saliva specimens from 72 patients with periodontitis, gingivitis, and healthy periodontal conditions were collected and analyzed, with no laboratory losses occurring during the process. The age and gender distribution percentages and mean values of the patient and control groups are demonstrated in Table 1.

Table 2 provides the periodontal parameters for the study groups. The values for the PI, PD in mm, CAL in mm, and BOP (%) were markedly higher in Group P than in both healthy and Group G ($p<0.001$). In the gingivitis group, periodontal parameters were markedly higher in comparison to those observed in the healthy group ($p<0.001$).

Table 1. The age and gender distribution percentages and mean values

	Periodontitis (n=24)	Healthy (n=24)	Gingivitis (n=24)
Gender, n (%)			
Male	10 (40.0%)	5 (20.0%)	9 (36.0%)
Female	15 (60.0%)	20 (80.0%)	16 (64.0%)
Age			
Mean (SD)	43.9 (11.71)	25.9 (6.03)	27.8 (6.80)

Table 2. The periodontal parameters for the study groups

Periodontal Parameters	Healthy (n=24)	Gingivitis (n=24)	Periodontitis (n=24)	P value
PI	0.56 ±0.03[a]	1.07±0.04[b]	1.93±0.04[b]	<0.001*
BOP (%)	2.02±0.1[a]	49.76±4.3[b]	52.40±14.3[b]	<0.001*
CAL	0.00±0.001[a]	0.45±0.002[a]	5.2±0.032[b]	<0.001*
PPD	1.92±0.02[a]	2.75±0.04[a]	3.76±0.05[b]	<0.001*

PI: Plaque Index, BOP: Bleeding on Probing, CAL: Clinical Attachment Level, PPD: Probing Pocket Depth.
[a,b,c] For the Kruskal-Wallis test, groups with a different superscript letter were statistically significant
* means statistical difference

Salivary miRNA-155 expression levels in the gingivitis patient group showed a statistically marginally significant decrease compared to the healthy group (Table 3, Figure 1). In contrast, the periodontitis group exhibited significantly higher expression levels compared to the healthy group (Table 3, Figure 1).

When all groups were analyzed collectively, a significant statistical difference was observed in miRNA-155 salivary expression levels (Table 3, Figure 1). MiRNA levels in the periodontitis group were notably elevated than those in the gingivitis and healthy groups ($p<0.05$).

Table 3. miRNA-155 expression levels

ΔCT	Periodontitis (n=24)	Gingivitis (n=24)	Healthy (n=24)	P-value
Mean (SD)	-2.1 (2.85)[a]	-3.7 (1.48)[b]	-2.9 (1.62)[c]	**0.0245***

ΔCT : Delta cycle threshold
[a,b,c] For the Kruskal-Wallis test, groups with a different superscript letter were statistically significant
* means statistical difference

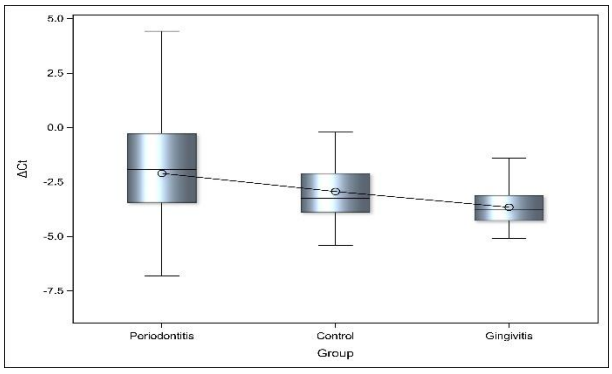

Figure 1.

In our study, sensitivity, and specificity calculations for various ΔCT threshold values in predicting disease status were performed, and the optimal threshold value for ΔCT was determined along with its corresponding sensitivity and specificity values. Additionally, an empirical ROC curve was generated using a non-parametric method with SAS software. For gingivitis, an AUC value of 0.61 was obtained with a 95% confidence interval (CI) of 0.4610–0.7774 and a $p<0.001$ (Table 4, Figure 2). For periodontitis, an AUC value of 0.63 was calculated with a 95% CI of 0.4701–0.7923 and a $p<0.001$ (Table 5, Figure 3). Both curves and their corresponding AUC values indicate that ΔCT has predictive capacity in distinguishing diseased individuals from healthy individuals.

Table 4. ROC Analysis of miRNA-155 ΔCT or Gingivitis

	Estimate	95% Confidence Limits	
PPV	0.6500	0.4409	0.8590
NPV	0.6000	0.4246	0.7753
Sensitivity	0.5200	0.3241	0.7158
Specificity	0.7200	0.5439	0.8960
AUC	0.6192	0.4610	0.7774
	cutoff	**prob**	**Youden**
deltaCT	-3.78	0.5374	0.24
	Somers' D	**Gamma**	**Tau-a**
	0.2384	0.2392	0.1216
True Positive	**True Negative**	**False Positive**	**False Negative**
13	18	7	12

PPV: Positive Predictive Value, NPV: Negative Predictive Value, AUC: Area Under the Curve

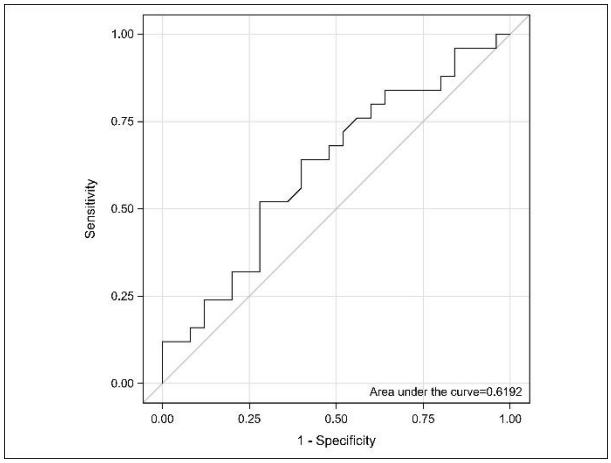

Figure 2.

Table 5. ROC Analysis miRNA-155 ΔCT for Periodontitis

	Estimate	95% Confidence Limits	
PPV	0.7222	0.5153	0.9291
NPV	0.6250	0.4572	0.7927
Sensitivity	0.5200	0.3241	0.7158
Specificity	0.8000	0.6432	0.9568
AUC	0.6312	0.4701	0.7923
	cutoff	**prob**	**Youden**
deltaCT	-1.91	0.52501	0.32
	Somers' D	**Gamma**	**Tau-a**
	0.2624	0.2628	0.1339
True Positive	**True Negative**	**False Positive**	**False Negative**
13	20	5	12

PPV: Positive Predictive Value, NPV: Negative Predictive Value, AUC: Area Under the Curve

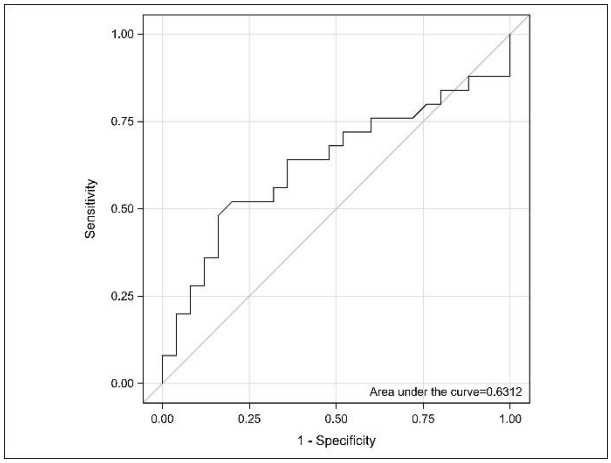

Figure 3.

DISCUSSION

Saliva is increasingly recognized as a valuable biofluid and source of biomarkers for the diagnosis of various dental and systemic conditions. The utilization of miRNAs in the non-invasive diagnosis and prognosis of periodontal diseases is emerging as a promising approach.[19]

In the periodontium, miRNAs serve critical regulatory functions in growth, maintaining periodontal balance, and disruption of periodontal tissue cohesion associated with the pathogenesis of PD. [3]

Infections with various pathogens induce the temporally regulated expression of specific microRNA subsets. Among these, miRNA-155 plays a pivotal role in modulating host cellular reactions to bacterial challenges and in regulating adaptive immunity, serving as a central regulator of T-cell responses.[7,20]

MiRNA-155 is regulated via toll-like receptors (TLRs) in inflammatory diseases, with all TLR signaling pathways converging on the activation of NF-κB, manages the expression of genes that are involved in producing cytokine related to inflammation. [21,22] TLRs are responsible for mediating innate immune responses through signaling pathways to ensure effective host defense against pathogenic infections.[23,24]

In our study, we observed that miRNA-155 expression is elevated during periodontitis and further increases as gingivitis progresses to periodontitis. Mogharehabed et al.'s study[25] found that miRNA-155 levels in tissue and blood samples from individuals with chronic periodontitis support our findings. They reported that miRNA-155 expression levels were significantly elevated in the patients with chronic periodontitis compared to the control group. Additionally, they stated a positive correlation between miRNA-155 levels and clinical parameters. In another study focused on gingival tissue samples, a five-fold increase in miRNA-155 expression was detected in the patient group than the control group.[11]

Al-Rawi et al.[26] reported that miRNA-155 was highly expressed in PD patients' saliva and that these expressions were observed to be positively correlated with the PD severity. They concluded that miRNA-155 might be crucial in the development and progression of PD.

In our study, we observed that patients in the gingivitis group had lower levels of miRNA-155 compared to the healthy control group. Given that gingivitis is a reversible PD and has not been thoroughly investigated in terms of miRNA expression levels, it is noteworthy that miRNA-155 levels were similar to those in the healthy group. It has been reported that miRNA-155 gene expression is increased in individuals with PD than in the control group and that it is significantly increased in the early-stage periodontitis group than in gingivitis group. [27]

MiRNAs are differentially expressed in cells induced by TLRs, which function as membrane-bound initiators

and trigger the inflammatory cascade.[28] Xie et al.[29] observed that, similar to our findings in gingivitis patients, Hsa-miR-155 expression in inflamed tissues was significantly lower compared to that in healthy control tissues. They suggested that this miRNA might be important in the pathogenesis of periodontal inflammation by regulating or interacting with TLR-associated pathways. It has been recorded that an anti-inflammatory cytokine IL-10 reduces the inflammatory response following pathogen invasion and protects the host from excessive inflammation, inhibits miRNA-155 expression in response to TLR4 stimulation. [30]

The detection of low levels of miRNA-155 in the gingivitis group may be attributed to the fact that this disease represents an earlier and reversible inflammatory process. Since gingivitis is not characterized by the more advanced inflammatory responses seen in chronic periodontitis, the suppressed expression of miRNA-155 may be associated with the milder course of the disease and the absence of tissue destruction. This suggests that miRNA-155 may be essential in the control and regulation of severe inflammation.

Additionally, miRNA-155 participates in inflammatory processes and the pathogenesis and molecular management of periodontal inflammation through its targets, such as suppressors of cytokine signaling and fibroblast growth factors.[31] The conflicting results between gingivitis and periodontitis can be attributed to dissimilarities in the characterization of cell types and histology of tissues examined, considering that gingival tissue includes various cell types, including fibroblasts or epithelial cells, adding significant complexity to the regulatory process.

Our research represents the pioneering study in literature to examine salivary miRNA-155 expression levels in individuals with both gingivitis and periodontitis. This comparison enhances the understanding of the role of miRNA-155 in periodontal inflammation and aids in assessing its potential as a biomarker for PD. Our findings indicate that miRNA-155 is associated with increased inflammation, with salivary levels being the lowest in gingivitis and the highest in periodontitis. These results suggest that miRNA-155 could be a biomarker correlated with the severity of inflammation and potentially a therapeutic target for PD.

A limitation of our study is the inability to determine expression levels post-treatment. However, we believe that this preliminary study will shed light on changes in miRNA-155 levels in a larger sample group and verify post-treatment changes by comparing them with miRNA levels in GCF or serum.

CONCLUSION

The role of miRNA-155 in PD is analyzed by comparing the miRNA-155 expression levels in the saliva of individuals with gingivitis and periodontitis. Our findings indicate that miRNA-155 is closely associated with periodontal inflammation and that its expression changes in parallel with the severity of inflammation. Notably, the significant difference in miRNA expression levels between gingivitis and periodontitis underscores the potential of this molecule as a biomarker for inflammation

The fact that miRNA-155, known to be closely related to physiological and pathological processes, can be detected in the saliva of individuals with PD through a non-invasive method, suggests that this molecule could be an important biomarker for early diagnosis as well as a potential therapeutic target. Future research is expected to contribute more robust evidence on the role of miRNA-155 in the early diagnosis and treatment strategies of PD, thereby making significant contributions to clinical applications in this field..

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