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Investigation of salivary miRNA-199a-5p levels in plaque-induced gingivitis

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ABSTRACT

Aims: Epigenetic mechanisms play a crucial role in regulating the response to chronic inflammation in periodontal diseases and serve as reliable biomarkers. While these biomarkers are traditionally assessed through invasive techniques, analyzing miRNA expression in saliva offers a non-invasive and convenient alternative. Our study aims to investigate salivary miRNA-199a-5p levels in both healthy and diseased conditions and to evaluate its potential role as an early diagnostic indicator of periodontal diseases such as gingivitis.

Methods: Saliva samples from 50 individuals, either diagnosed with gingivitis or exhibiting a healthy periodontal condition, were analyzed using miRNeasy serum/plasma kit (Qiagen). SPSS software was used for statistical analysis. MiRNA-199a-5p expression levels were compared using the Mann-Whitney U test.

Results: In saliva samples collected from 50 individuals, the expression levels of miRNA-199a-5p were significantly elevated in the gingivitis group compared to the healthy control group. Aditionally, the bleeding on probing (BOP %) values were significantly higher in group G compared to the healthy group.

Conclusion: miRNA-199a-5p can be considered an important regulator with potential as both an early diagnostic biomarker and a therapeutic target for periodontal diseases. Although its role in autophagy and inflammation shows promise, further studies are needed to clarify its specific functions and clinical relevance.

Keywords: miRNA-199a-5p, gingivitis, periodontal disease

INTRODUCTION

The natural microbiota of the oral cavity tends to form a polymicrobial biofilm on the mineralized surfaces of teeth, particularly in the presence of periodontal disease. This biofilm exerts detrimental effects on periodontal tissue health.[1] As the dental biofilm matures, an inflammatory response is triggered within the gingival tissue, manifesting clinically as an increased flow of gingival crevicular fluid (GCF), gingival bleeding, and edema.[2] In 1965, Löe and colleagues[3] conducted the classic study titled "experimental gingivitis in man," demonstrating that the accumulation of supragingival biofilm leads to gingival inflammation, which resolves upon its removal. Yamamoto and his team elaborated on gingivitis, defining it as a nonspecific and reversible inflammatory condition that arises due to an increase in gram-negative or gram-positive bacterial populations within or below the gingival sulcus.[4]

MicroRNAs (miRNAs) are short, single-stranded RNA sequences that do not encode proteins and typically comprise around 22 nucleotides. More than 1.000 miRNAs have been identified in the human genome, and they regulate gene expression by binding with the 3'-untranslated region (3'-UTR) of designated target genes.[5,6] Following transcription, miRNAs can bind to complementary mRNA sequences, thereby suppressing, activating, or modulating protein translation and synthesis.[7,8] The relationship between miRNAs and different pathologies has been reported in detail. A single miRNA has the ability to regulate the expression of hundreds of target genes at the same time.[9] Their presence in various

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tissues and bodily fluids, along with their high stability in biological fluids, has led to the recognition of miRNAs as potential biomarkers in different clinical settings.[7]

miRNAs play a crucial role in controlling cellular proliferation, development, differentiation, carcinogenesis, and inflammation. Additionally, miRNA profiles have been compared between healthy and periodontally diseased conditions.[10,11] miRNA-199 targets multiple genes, including TNF-alpha, prostaglandins, IL-1β, IL-6, BMP-2, EGF, and TGF-β.[12,13] Notably, miRNA-199a-5p overexpression disrupts autophagy and activates the mTOR/GSK3β pathway.[14]

The mammalian target of rapamycin (mTOR) plays a crucial role in two distinct protein complexes, mTORC1 and mTORC2, which regulate various cellular processes.[15] It promotes anabolic metabolism while inhibiting autophagy, the primary cellular degradation process responsible for eliminating damaged macromolecules and organelles.[16] Upon mTOR inhibition, autophagic degradation and recycling of cellular components contribute to the reactivation of mTORC1. As a result, autophagy plays a regulatory role in both the upstream and downstream pathways of mTOR signaling.[17]

The studies also demonstrated that hsa-miRNA-199a plays a functional role as a direct inhibitor of IKKβ expression, a key upstream activator of NF-κB. Furthermore, MAPK4 has been identified as a direct target of miRNA-199a-5p, and its inhibition was associated with reduced inflammation and decreased production of proinflammatory cytokines.[18,19]

In this context, several studies have proposed the potential use of miRNA-199a-5p as a biomarker due to its presence during early inflammatory changes.[20,21]

Epigenetic mechanisms in periodontal diseases are considered reliable biomarkers that regulate adaptation to chronic inflammatory stimuli. Although the expression levels of these biomarkers are typically analyzed using invasive methods, investigating miRNA expression in saliva provides a non-invasive and more accessible alternative.[22-24]

A better understanding of the expression dynamics of miRNA-199a-5p may contribute to the development of novel, non-invasive diagnostic approaches for periodontal health monitoring. Accordingly, our study aims to investigate miRNA-199a-5p expression levels under both healthy and diseased conditions and to evaluate its potential role as an early diagnostic biomarker for periodontal diseases such as gingivitis.

METHODS

Ethics

This research was carried out in compliance with the principles outlined in the Declaration of Helsinki. Ethical approval for this study was obtained from the Non-interventional Clinical Researches Ethics Committee of Hitit University (Date: 05.12.2024, Decision No: 323/2024-25).

Clinical Samples

To determine the required sample size, a statistical power analysis was conducted using G*power version 3.1. Based on an effect size of 0.25 and a power of 90%, the total required sample size was calculated. The study was conducted with 50 participants, divided equally into two groups (n=25), consisting of patients admitted to the Faculty of Dentistry at Akdeniz University and healthy individuals attending routine dental assessments.

Participants were required to have no systemic diseases, no current medication use, no crowns, veneers, or restorations, and at least 20 teeth retained in the oral cavity, and to be non-smokers. Exclusion criteria included the presence of systemic diseases, pregnancy, menstrual periods, periodontal treatment within the last six months, or the use of anti-inflammatory drugs or antibiotics within the past two weeks.

Subjects were divided into two groups according to their periodontal condition: the gingivitis group (group G) and the healthy periodontium group (group H). The definitions of gingivitis and health were established according to the 2017 revised Classification of Periodontal and Peri-Implant Diseases and Conditions by the World Workshop on Periodontology.[25]

Group H consisted of individuals exhibiting a clinically healthy periodontal status. Group G comprised individuals who did not exhibit radiographic evidence of clinical attachment loss (CAL) or alveolar bone loss but had BOP scores of 10% or higher. Panoramic radiographs were used to assess alveolar bone loss. Both the clinical examination and periodontal measurements were performed by periodontologists. CAL, probing depth (PD), and plaque index (PI) were evaluated using a periodontal probe at six designated locations on each tooth. The PI was determined based on the criteria established by Silness and Löe[26] CAL measurements were conducted to maintain periodontal status consistency among participants, confirm the absence of attachment loss in the periodontally healthy and gingivitis groups, and comprehensively characterize the clinical and demographic profiles of the study population in accordance with established periodontal research protocols. CAL assessments also served to accurately identify pure gingivitis cases and to exclude participants with any suspicion of prior periodontitis.

Laboratory Measurements

Non-stimulated saliva specimens were obtained for research. Participants were advised to abstain from food, beverages, and gum chewing for a minimum of one hour prior to sample collection. Saliva specimens were obtained under controlled environmental conditions between 09:00 and 11:00 AM, transferred into plastic containers, and then transferred into centrifuge tubes using sterile syringes. To enhance the accuracy of the analysis and reduce turbidity, saliva samples were vortexed at room temperature for 10 minutes to remove cellular debris and food particles. The supernatant was carefully collected and stored at –80°C until miRNA analysis. The samples were transported on dry ice to İstanbul Okan University, where the analyses were performed.

miRNA isolation: On the day of the experiment, saliva samples were thawed and centrifuged at 11.000 g at +4°C for 20 minutes. miRNA isolation was then performed from the supernatant using the miRNeasy serum/plasma kit (Cat. No./ID: 217184, Qiagen, Hilden, Germany) according to the

manufacturer's protocol. The purity and concentration of the isolated miRNAs were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

cDNA synthesis: Complementary DNA (cDNA) synthesis was performed using the miRCURY LNA RT Kit (Cat. No./ID: 339340, Qiagen, Germany). During the cDNA synthesis process, the isolated miRNA samples underwent reverse transcription.

Determination of miRNA levels using fluorometry: The concentration of transcribed miRNAs was measured using a Qubit 3.0 Fluorometer (Thermo Scientific) following the standard protocol of the Qubit miRNA Assay Kit. After concentration measurements, appropriate dilutions were made.

miRNA expression analysis: Following the quantification of sample concentrations, miRNA-199a-5p expression levels (miRCURY Lot: 201803080050-4, Qiagen, Germany) were measured using the Rotor-Gene Q Real-Time PCR System (Rotor-Gene Q, Qiagen) with the miRCURY LNA SYBR Green PCR Kit (Cat. No./ID: 339346, Qiagen, USA).

The housekeeping gene (RNU6, Lot: 20800469-1, Qiagen) was utilized as an internal reference for normalization of ΔCT values and to calculate the fold change in miRNA expression levels. The Livak formula ($2^{-\Delta\Delta C}{}_T$) was applied to determine miRNA levels. The ΔΔCT value was calculated by subtracting the ΔCT of the target gene from the mean ΔCT of the internal control, and the fold change was subsequently computed as $2^{-\Delta\Delta C}{}_T$.[27]

Statistical Analysis

The data analyses of the study were carried out using SPSS software version 26.0. The Kolmogorov-Smirnov test was employed to evaluate the data's normality, while variance homogeneity was analyzed using Levene's test. Differences in PI, gingival index, and PD among study groups were evaluated using one-way analysis of variance (ANOVA) followed by post-hoc Tukey tests. The miRNA-199a-5p levels between study groups were compared using the Mann-Whitney U test. Statistical significance was set at $p<0.05$ with a 95% confidence interval.

RESULTS

In our investigation, saliva samples from 50 individuals, either diagnosed with gingivitis or exhibiting a healthy periodontal condition, were analyzed. No laboratory losses occurred during the process. The age and gender distribution percentages and mean values of the patient and control groups are presented in **Table 1**.

Periodontal parameters for the study groups are displayed in **Table 2**. While no statistically significant differences were observed in PI, probing depth (PD in mm), or clinical attachment loss (CAL in mm), the bleeding on probing (BOP in %) values were significantly higher in group G compared to the healthy group ($p<0.001$). Additionally, salivary miRNA-199a-5p expression levels in the gingivitis group were significantly higher than those in the healthy group (**Table 3, Figure 1**).

Table 1. Demographic characteristics

	Healthy (n=25)	Gingivitis (n=25)
Age		
Mean (SD)	25.9 (6.03)	27.8 (6.80)
Median (range)	23.0 (19.0, 42.0)	26.0 (18.0, 46.0)
Gender, n (%)		
Male	5 (20.0%)	9 (36.0%)
Female	20 (80.0%)	16 (64.0%)

SD: Standard deviation

Table 2. Periodontal parameters

Periodontal parameters	Healthy	Gingivitis	p value
PI	0.68±0.02	1.19±0.03	<.001
PD (mm)	1.81±0.02	2.14±0.02	<.001
BOP (%)	2.13±0.2	46.93±5.5	<.001
CAL (mm)	0.00±0.002	0.58±0.001	<.001

PI: Plaque index, PD: Probing depth, BOP: Bleeding on probing, CAL: Clinical attachment loss

Table 3. miRNA-199a-5p 2−ΔΔCT values

	Group		
	Gingivitis (n=25)	Healthy (n=25)	p-value
$2^{-\Delta\Delta C}{}_T$			0.0008
Mean (SD)	33.3 (4.01)	29.3 (4.04)	
Median (range)	33.7 (22.3, 37.9)	29.3 (22.3, 36.5)	

SD: Standard deviation

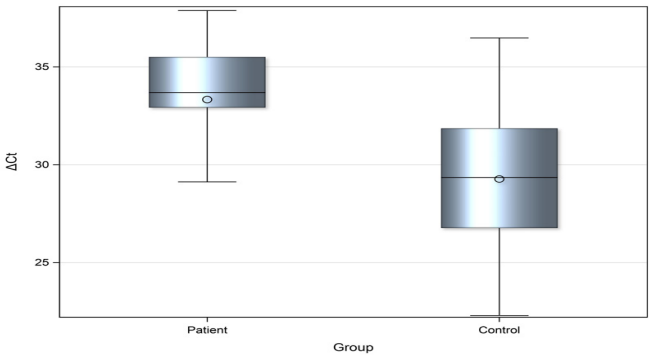

Figure 1. Graphical representation of miRNA199-5p levels in patient and control groups

In our study, sensitivity and specificity calculations were performed for various ΔCT threshold values to predict disease status, and the optimal threshold value for ΔCT was determined along with its corresponding sensitivity and specificity values. An empirical receiver operating characteristic (ROC) curve was generated using a non-parametric method with SAS software. For gingivitis, an area under the curve (AUC) value of 0.77 was obtained, with a 95% confidence interval (CI) of 0.60728–0.93118 and a p-value <0.001 (**Table 4, Figure 2**).

DISCUSSION

Periodontal tissue undergoes continuous remodeling due to tissue regeneration, mechanical stress, and inflammatory components arising from periodontal diseases.

Table 4. ROC analysis of miRNA-199a-5p for gingivitis

	Estimate	95% Confidence limits	
PPV	0.76923	0.60728	0.93118
NPV	0.79167	0.62919	0.95415
Sensitivity	0.80	0.64320	0.95680
Specificity	0.76	0.59258	0.92742
AUC	0.7760	0.60728	0.93118
	Cutoff	**Prob**	**Youden**
$2^{-\Delta\Delta C}{}_{T}$	32.77	0.57903	0.56
	Somers' D	Gamma	Tau-a
	0.5520	0.5538	0.2816
True positive	True negative	False positive	False negative
20	19	6	5

ROC: Receiver operating characteristic, AUC: Area under the curve

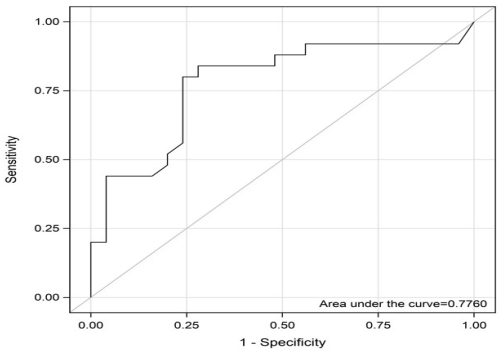

Figure 2. Receiver operating characteristic curve for miRNA199-5p

Biomarkers can be valuable in overcoming challenges encountered in preventive, predictive, and personalized healthcare applications across various medical fields. Therefore, miRNAs may serve as a key factor in the early diagnosis and prognosis prediction of various periodontal diseases.[7] Biological fluids are proposed as a significant research source for miRNA biomarkers. This is attributed to the efficient isolation and detection of miRNAs through quantitative polymerase chain reaction (qPCR), the minimally invasive nature of GCF and saliva sampling, and the high stability of miRNAs across various biological fluids.[28,29]

miRNAs regulate periodontal remodeling and inflammation by modulating key factors involved in osteogenesis (e.g., Bone Morphogenetic Protei-7, Runt-related transcription factor 2, osterix, osteocalcin), osteoclastogenesis (e.g.,Nuclear Factor of Activated T-cells, cytoplasmic 1, NF-κB, Receptor Activator of Nuclear Factor κB Ligand), and inflammatory mediators (e.g., IL-1β, IL-6, IL-8, TNF-α).[30]

The signaling pathways influenced by miRNA-199a-5p can modulate the progression from gingivitis to periodontitis through distinct mechanisms. Among these, the NF-κB pathway plays a pivotal role in orchestrating inflammatory processes by regulating proinflammatory cytokine production, leukocyte recruitment, and cell survival. Intriguingly, the functional outcome of NF-κB activation appears to be highly context-dependent, with its signaling axis capable of either sustaining the inflammatory milieu or promoting the resolution of inflammation through the induction of leukocyte apoptosis.[31] Considering the complexity of the pathways regulated by miRNA-199-5p, it is critical that future studies aim to elucidate their specific roles in the initiation, perpetuation, and resolution of inflammatory diseases.

Given their central role in regulating osteogenesis, osteoclastogenesis, and inflammatory pathways, miRNA-199-5p, could be considered potential biomarkers for the diagnosis and prognosis of periodontal disease. Moreover, the ability to detect these miRNAs in saliva further supports their applicability as non-invasive diagnostic tools.

Previous studies have demonstrated that miRNA-199a-5p is associated with a range of inflammatory disorders, cardiovascular diseases, and stork.[32,33] A review of the existing literature indicates that this study is the first to analyze the expression of miRNA-199a-5p in plaque-induced gingivitis. In this study, salivary miRNA-199a-5p levels in plaque-associated gingivitis were compared with those in individuals with clinically healthy periodontium. The results demonstrated a statistically significant increase in miRNA-199a-5p expression levels in the gingivitis group.

A previous study found that individuals with severe periodontitis exhibited an increased release of miRNA-199a-3p in GCF, which was positively correlated with the amount of GCF compared to individuals with a healthy periodontium.[34] In another study on salivary gland tumors, miRNA-199a was significantly upregulated in serum samples from the malignant group compared to the benign group.[35]

Yuan et al.[36] reported that miRNA-199-5p overexpression significantly suppresses oral fibroblasts proliferation, whereas the inhibition of miRNA-199-5p may lead to increased cell proliferation. Moreover, studies have shown that smokeless tobacco (SLT) users have a higher likelihood of developing periodontitis.[37] Analysis of saliva samples from SLT users revealed a significant increase in miRNA-199a-3p expression compared to non-tobacco users.[38]

Li et al.[39] suggested that increased miRNA-199-5p expression in patients with oral lichen planus (OLP) may contribute to OLP development by modulating mTOR and AKT1 secretion, proposing miRNA-199-5p as a potential therapeutic target. Similarly, Wang et al.[40] reported that miRNA-199 levels were lower in OLP patients compared to the control group. Luminescence assays confirmed that miRNA-199 directly suppressed mTOR secretion. Furthermore, the AKT/mTOR pathway was shown to regulate the autophagy mechanism, contributing to OLP pathogenesis.[40] These findings emphasize the need for more comprehensive studies to better understand the relationship between miRNA-199, OLP, and autophagy.

Increased mTORC1 activity can directly stimulate the maturation of pro-inflammatory T cells in periodontal diseases by enhancing glycolytic flux. Since autophagy is inhibited by mTORC1, these findings suggest that the detrimental effects of mTOR signaling on periodontal diseases may occur through autophagy regulation.[15]

Beyond the autophagy-related mechanisms involving mTORC1, recent evidence highlights the critical role of

miRNA-199a-5p in modulating inflammatory responses and structural alterations in periodontal tissues. miRNA-199a-5p, identified as a key regulator of intercellular junctions, functions as a proinflammatory miRNA, and its elevated expression in gingival tissues, GCF, saliva, and plasma has been associated with the presence and/or severity of periodontal disease.[19,41] These alterations represent crucial stages in the progression from gingivitis to periodontitis.

Gingivitis is a multifactorial, preventable, and treatable disease that develops in response to plaque biofilm. However, if inflammation persists, gingivitis can progress into periodontitis, a chronic inflammatory disease characterized by bone loss, affecting 45–50% of adults worldwide and expected to increase in prevalence.[42]

Given its detectable expression levels in saliva and its association with early inflammatory changes, miRNA-199-5p could serve as a non-invasive biomarker for the early diagnosis of periodontal disease and for monitoring treatment responses. Incorporating miRNA-based diagnostics into routine periodontal assessments may facilitate earlier intervention and improve clinical outcomes.

Limitations

Although the a priori power analysis conducted in this study demonstrated that the number of participants was statistically sufficient, the absence of previous studies specifically investigating miRNA-199-5p in gingivitis patients limits the generalizability of the findings. Therefore, while the current statistical design provides a robust foundation, larger-scale studies are needed to validate and expand these preliminary results across broader populations.

CONCLUSION

miRNA-199-5p emerges as a key regulatory molecule, both as a predictive biomarker for the early recognition of periodontal diseases and as a potential therapeutic target. While studies investigating its effects on autophagy and inflammation are encouraging, further research is required to elucidate its precise functions and clinical significance.

As one of the first studies focusing on the relationship between miRNA-199-5p, autophagy, and periodontal disease, this research aims to contribute to future scientific investigations. We believe that the findings will support the development of new diagnostic and therapeutic approaches through studies conducted on larger patient cohorts.

ETHICAL DECLARATIONS

Ethics Committee Approval

Ethical approval for this study was obtained from the Non-interventional ClinicalResearches Ethics Committee of Hitit University (Date: 05.12.2024, Decision No: 323/2024-25).

Informed Consent

All patients signed and free and informed consent form.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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