Tohoku J. Exp. Med., 2024, **264**, 121-130

Expression Levels of miR-181 Family Members in Oral Biofluids as Biomarkers for Periodontitis Severity

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This study aimed to assess the diagnostic potential of microRNA-181 (miR-181) family members in oral biofluids, namely saliva and gingival crevicular fluid (GCF), as biomarkers for periodontitis severity. A cohort of 150 patients with periodontitis, including 82 with mild to moderate and 68 with advanced periodontitis, along with 90 healthy controls, were recruited. Analysis of miR-181 family expression using quantitative real-time polymerase chain reaction (qRT-PCR) revealed differential expression levels in oral biofluids among the study groups. Salivary miRNAs, particularly miR-181a, displayed significant discriminatory ability in distinguishing periodontitis patients from healthy controls and between different stages of periodontitis severity, with high sensitivity and moderate to high specificity. In GCF samples, miR-181a and miR-181b exhibited robust discriminatory ability, while miR-181c showed moderate discriminatory ability. Conversely, miR-181d demonstrated lower discriminatory power in both saliva and GCF. Additionally, combination diagnosis using miR-181 family showed superior performance compared to individual miRNAs. Furthermore, enzyme-linked immunosorbent assay (ELISA) analysis of inflammatory biomarkers (TNF-α, IL-6, and IL-1β) in GCF revealed elevated levels in periodontitis patients compared to healthy controls, with a further increase observed in advanced periodontitis. Spearman correlation analysis demonstrated a significant negative correlation between miR-181 family expression in GCF and inflammatory biomarker levels, indicating their potential role in modulating periodontal inflammation. Overall, these findings suggest that miR-181 family members in oral biofluids, particularly saliva, hold promise as diagnostic biomarkers for periodontitis severity. Additionally, their negative correlation with inflammatory biomarkers highlights their potential as modulators of periodontal inflammation, providing valuable insights into the pathogenesis of periodontitis.

Keywords: biomarkers; gingival crevicular fluid; microRNAs; periodontitis; saliva.
Tohoku J. Exp. Med., 2024 November, **264** (3), 121-130.
doi: 10.1620/tjem.2024.J058

Introduction

Periodontitis, characterized by chronic inflammation and irreversible damage to the supporting tissues of teeth, is a significant global oral health concern, affecting 7%-11% of adults in severe cases (Grant et al. 2022; Verma et al. 2023). While biofilm formation is the primary cause, various host-related factors, including genetics, immunological, and environmental factors, contribute to its complexity and challenge early diagnosis (Sanchez-Medrano et al. 2023). The lack of consistent clinical guidelines in periodontology further complicates the detection of disease progression, treatment efficacy assessment, and determination of patient susceptibility (Bagde et al. 2023). Timely detection and effective management of periodontitis are crucial to prevent its advancement and associated complications.

MicroRNAs (miRNAs), distinguished by their stability, tissue-specific expression patterns, and regulatory roles in gene expression, have emerged as promising biomarkers for various diseases, including periodontitis, owing to their distinct expression profiles in different disease states (Daily et al. 2023; Safari et al. 2023). With advancements in highly sensitive protein assays, whole saliva and gingival crevicular fluid (GCF) have emerged as valuable sources of biomarkers with diverse applications in dentistry and medicine, commonly employed for monitoring health status and aiding disease diagnosis (Papagerakis et al. 2019). For instance, miR-103a-3p, miR-423-5p, miR-23a-3p, miR-

Received March 28, 2024; revised and accepted June 22, 2024; J-STAGE Advance online publication July 4, 2024
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15a-5p, and miR-223-3p in GCF exhibit correlations with each other and with inflammatory mediators, suggesting their potential as biomarkers for different stages of periodontitis and as therapeutic targets (Costantini et al. 2023). Additionally, salivary miRNAs, including miR-5571-5p, let-7f-5p, miR-99a-5p, miR-28-5p, and hsa-miR-320d, demonstrated potential as biomarkers for predicting the progression of chronic periodontitis (Fujimori et al. 2021).

The miR-181 family, a highly conserved group of miRNAs expressed in numerous tissues and cell types, consists of miR-181a/b/c/d (Jimenez et al. 2022; Bell-Hensley et al. 2023). This family has garnered attention as a significant contributor to inflammatory processes in multiple physiological contexts (Jimenez et al. 2022). Furthermore, circulating miR-181 has emerged as a promising diagnostic biomarker for various diseases, such as schizophrenia, amyotrophic lateral sclerosis and cancers (Rizos et al. 2015; Pop-Bica et al. 2018; Magen et al. 2021; Autenshlyus et al. 2023). Studies have highlighted that several miRNAs within the miR-181 family, such as miR-181a, miR-181b, and miR-181c, are downregulated in diseased periapical tissues compared to healthy ones, along with significant downregulation observed in inflamed human pulps relative to normal pulps (Zhong et al. 2012; Chan et al. 2013). Additionally, a study elucidated the regulatory role of miR-181a in modulating IL-8 expression in TLR4/2+ primary human dental pulp fibroblasts stimulated with lipopolysaccharide from *Porphyromonas gingivalis* (Pg LPS), highlighting its significance in the inflammatory response associated with dental pulp inflammation (Galicia et al. 2014). These findings imply that the dysregulation of miR-181 family members may play a role in the pathogenesis of inflammatory conditions such as periodontitis.

Therefore, this study aims to explore the potential of miR-181 family microRNAs in oral fluids (saliva and GCF) as promising biomarkers for chronic periodontitis and to provide insights into their utility for precision medicine and improved diagnostics in periodontal care.

Methods and Materials

Subject population

The study recruited individuals aged 18 years or older, in good general health, and with a minimum of 20 teeth. Before the clinical examination, participants completed a detailed questionnaire covering demographic information and systemic health status, including any history of diabetes or other systemic diseases. Exclusion criteria included current smokers or those who had quit smoking within the last 5 years, individuals wearing orthodontic appliances, undergoing long-term antibiotic/anti-inflammatory therapy, pregnant or breastfeeding women, and those with medical/dental conditions incompatible with the study. The study was approved by the ethics committee of the China Resources and WISCO General Hospital, and written informed consent was obtained from all participants prior to enrollment.

Clinical examination

Periodontal clinical parameters were assessed in a standardized setting (using natural light) following established methodology and equipment. Parameters included clinical attachment loss (CAL), probing pocket depth (PPD), bleeding on probing (BOP), and the count of missing teeth due to periodontitis. Diagnosis and severity staging of periodontal disease were conducted according to the guidelines of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions (Tonetti et al. 2018a,b). The study comprised 150 patients with periodontal disease, including 82 with mild to moderate periodontitis (Stage I/II) and 68 with advanced periodontitis (Stage III/IV), as well as 90 healthy controls who exhibited no CAL, PPD $\leq$ 3 mm across all sites, and less than 10% of sites showing BOP.

Samples collection

Participants were asked to refrain from brushing, eating, or drinking (except water) for at least 2 h before sample collection. Saliva samples were collected from participants after they rinsed their mouths with water at the beginning of the appointment. Participants spat into containers for 10 minutes, yielding approximately 6 ml of whole saliva. The containers were kept on ice during collection and transportation to the laboratory. Saliva samples were centrifuged at 2,600 × *g* for 15 minutes at 4°C. The supernatant was collected in new tubes containing a protease inhibitor solution and stored at −80°C. After saliva was collected, GCF was also collected using microcapillary pipettes for all participants during the same visit. For participants with periodontitis, GCF was collected at a predetermined site with periodontal probing depth > 4 mm, while for periodontally healthy participants, GCF collection was conducted on the palatal side of maxillary incisors (Rovas et al. 2021). After isolating the area with a lignin roll and removing supragingival plaque, the pipette was placed extracrevicularly against the tooth for 5 minutes, collecting 1 μL from each site. The GCF samples were immediately ejected into test tubes, mixed with phosphate buffer solution, and frozen at −80°C for storage. The human tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-1β concentrations in GCF samples were measured by Enzyme-linked immuno sorbent assay (ELISA, Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR

RNA extraction from saliva and GCF samples was conducted using the commercially available TaqMan miRNA Reverse Transcription kit. The quantity and purity of the extracted RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, reverse transcription was carried out utilizing the TaqMan® MicroRNA Reverse Transcription Kit to synthesize cDNA. Real-time PCR analysis was performed using the TaqMan® MicroRNA

Assays Kit on an Applied BioSystems 7900HT thermocycler (Applied Biosystems. Inc, CA, USA). To ensure normalization, cel-miR-39 was spiked in as an internal control. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Data analysis was conducted with GraphPad Prism 8.0. Categorical variables were expressed as counts (n) and analyzed using the chi-square test. Continuous variables were presented either as mean ± SD for normally distributed data or as median with interquartile range (IQR) for non-normally distributed data, determined by the Shapiro-Wilk test. The comparisons of measurement data were conducted using ANOVA followed by Holm-Sidak's multiple comparisons test or Kruskal-Wallis test followed by Dunn's multiple comparisons test. Diagnostic value was evaluated using ROC curve analysis (MedCalc software). The DeLong test was used to compare the area under the ROC curve (AUROC). Correlation between inflammatory biomarkers and miR-181 family expression in GCF from periodontitis patients was assessed using Spearman analysis. A P value < 0.05 was considered statistically significant.

Results

Baseline Characteristics and Clinical Parameters in Study Groups

A total of 150 patients with periodontitis were included, comprising 82 with mild to moderate periodontitis and 68 with advanced periodontitis, in addition to 90 healthy controls. Baseline characteristics revealed similar distributions of age, gender, and BMI across the groups (all $P > 0.05$, Table 1). Significant differences in clinical parameters among groups were observed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Healthy controls showed no CAL, while those with mild to moderate periodontitis had a median CAL of 2.45 mm (IQR 1.6-3.2), and individuals with advanced periodontitis exhibited substantially higher CAL values at 7.1 mm (IQR 6.1-7.875) ($P < 0.001$). Tooth loss attributed to periodontitis was absent in healthy controls and mild to moderate periodontitis but present in advanced periodontitis (median 5, IQR 4-6) ($P < 0.001$). Moreover, PPD (mm) was notably higher in both periodontitis groups (mild to moderate: median 4.1, IQR 3.5-4.7; advanced: median 7, IQR 6.7-7.5) compared to healthy controls (median 1.6, IQR 0.8-2.2, $P < 0.001$), and BOP (%) was significantly elevated in periodontitis groups (mild to moderate: median 21, IQR16-27; advanced: median 38, IQR 34.25-42) compared to healthy controls (median 4, IQR 2-8, $P < 0.001$).

Differential Expression of miR-181 Family in Periodontitis Patients

The miR-181 family's expression in periodontitis patients and healthy controls was compared using one-way ANOVA, followed by Holm-Sidak's multiple comparisons test to determine differential expression. As shown in Fig. 1, the expression levels of miR-181a, miR-181b, and miR-181c in oral biofluids (saliva and GCF) differed significantly among the three group, indicating an association with the severity of periodontitis (all $P < 0.05$). Additionally, miR-181d expression in oral biofluids was lower in both mild to moderate periodontitis and advanced periodontitis groups compared to healthy controls (all $P < 0.05$). However, no significant difference in miR-181d expression was observed between the mild to moderate periodontitis and advanced periodontitis groups in GCF ($P > 0.05$). Notably, in saliva samples, miR-181d expression exhibited differences only between healthy controls and advanced periodontitis patients ($P < 0.05$).

Salivary miR-181 Family MicroRNAs as Biomarkers for Periodontitis Severity

The analysis of ROC curves demonstrated the discriminatory performance of salivary miRNAs in distinguishing periodontitis patients from healthy controls, as well as across different stages of periodontitis severity (Table 2 and Fig. 2A). MiR-181a exhibited substantial discriminatory ability across all comparisons, with AUC values ranging

Table 1. Baseline Characteristics and Clinical Parameters in Study Groups.

	Healthy controls (n = 90)	Mild to moderate periodontitis (n = 82)	Advanced periodontitis (n = 68)	*P*
Age (years)	50 (44.75-59)	48.5 (42.75-55.25)	52.5 (44.25-59)	0.088
Gender				
Male	54	40	38	
Female	36	42	30	0.331
BMI (kg/m^2)	20.45 (19.2-22.33)	20.75 (19.18-22.73)	21.4 (19.93-23.25)	0.140
Clinical attachment loss (CAL, mm)	0	2.45 (1.6-3.2)*	7.1 (6.1-7.875)*,#	< 0.001
Tooth loss due to periodontitis	0	0	5 (4-6)*,#	< 0.001
Probing pocket depth (PPD, mm)	1.6 (0.8-2.2)	4.1 (3.5-4.7)*	7 (6.7-7.5)*,#	< 0.001
Bleeding on probing (BOP, %)	4 (2-8)	21 (16-27)*	38 (34.25-42)*,#	< 0.001

* indicates significance compared to healthy controls ($P < 0.05$), # indicates significance compared to mild to moderate periodontitis ($P < 0.05$).

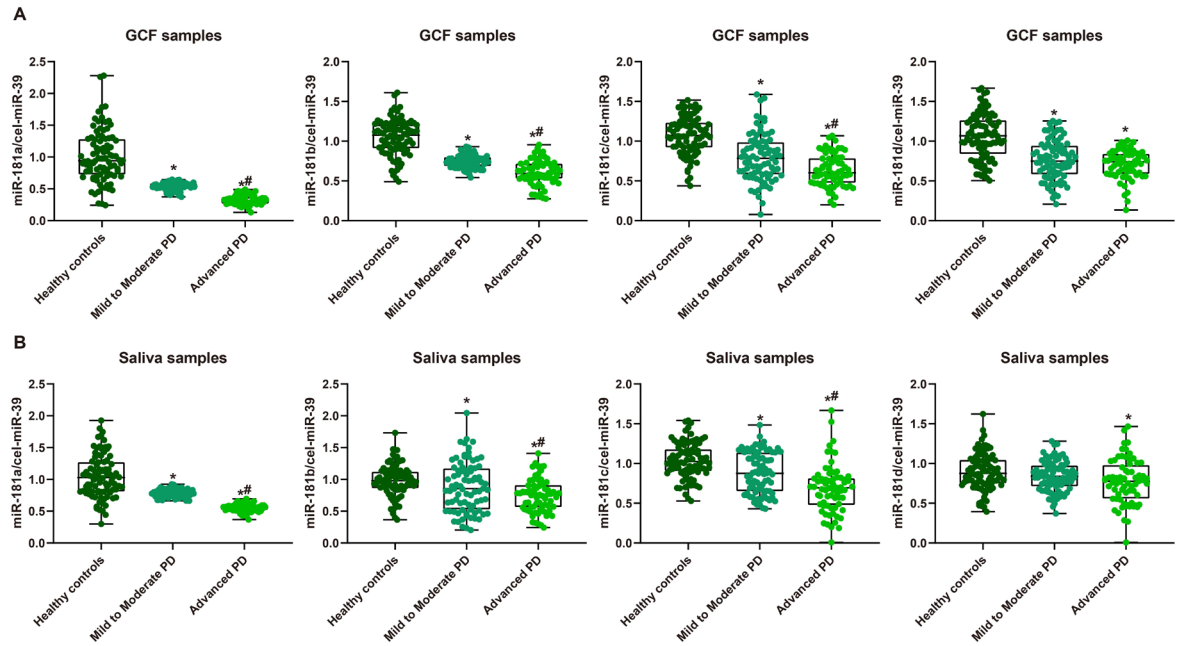

Fig. 1. Differential expression of miR-181 family in periodontitis patients.
Differential expression of miR-181 family (miR-181a/b/c/d) in gingival crevicular fluid (GCF) samples (A) and saliva samples (B) among patients with different severity of periodontitis (PD) and healthy controls. * indicates significance compared to healthy controls ($P < 0.05$), # indicates significance compared to mild to moderate periodontitis ($P < 0.05$).

from 0.854 to 0.999. Additionally, miR-181a displayed high sensitivity (ranging from 96.34% to 100%) and moderate to high specificity (ranging from 66.67% to 100%) in identifying periodontitis cases (all $P < 0.001$). MiR-181c also demonstrated promising discriminatory ability (all $P < 0.001$), particularly in distinguishing advanced periodontitis from HC (AUC = 0.845), with sensitivity of 82.35% and specificity of 81.11%, and between advanced and mild to moderate periodontitis (AUC = 0.687), with sensitivity of 79.41% and specificity of 56.1%. MiR-181b showed moderate discriminatory performance, with sensitivity ranging from 51.22% to 85.29% and specificity ranging from 39.02% to 80.00%, while miR-181d exhibited limited discriminatory power in most comparisons, with sensitivity ranging from 26.67% to 92.68% and specificity ranging from 21.11% to 90%. The combined diagnostic potential of miR-181 family microRNAs demonstrated superior performance compared to individual miRNAs, achieving high AUC values across all comparisons: 0.894 for periodontitis vs. healthy controls, 0.828 for mild to moderate periodontitis vs. healthy controls, 0.971 for advanced periodontitis vs. healthy controls, and 1.000 for advanced vs. mild to moderate periodontitis (all $P < 0.001$). Sensitivity ranged from 92.68% to 100%, and specificity ranged from 67.78% to 100%.

Statistical analysis using the DeLong test revealed significant differences in AUROC among miR-181 family in various comparisons. miR-181a vs. miR-181b/ miR-181d exhibited significant differences in all comparisons (all $P < 0.05$). Similarly, miR-181a vs. miR-181c showed significant differences in periodontitis vs. healthy controls ($P = 0.011$), advanced periodontitis vs. healthy controls ($P = 0.013$), and advanced vs. mild to moderate periodontitis ($P < 0.001$). Furthermore, significant differences were noted for miR-181a vs. combined in periodontitis vs. healthy controls ($P = 0.007$) and advanced periodontitis vs. healthy controls ($P = 0.029$). Comparisons between miR-181b vs. combined showed significant difference (all $P < 0.05$). miR-181c vs. miR-181d revealed significant differences for periodontitis vs. healthy controls ($P = 0.002$) and advanced periodontitis vs. healthy controls ($P < 0.001$). Lastly, miR-181c vs. combined and miR-181d vs. combined showed significant differences in all comparisons, with P-values indicating the diagnostic superiority of the combined miRNAs.

MiR-181 Family MicroRNAs in GCF as Biomarkers for Periodontitis Severity

As demonstrated in Table 3 and Fig. 2B, miR-181a expression in GCF demonstrated robust discriminatory ability across all comparisons, with AUC values ranging from 0.806 to 0.984 (all $P < 0.001$). MiR-181b also showed promising discriminatory performance, with AUC values ranging from 0.776 to 0.941 (all $P < 0.001$). Furthermore, miR-181c exhibited moderate discriminatory ability, with AUC values ranging from 0.667 to 0.915. Conversely, miR-181d displayed lower discriminatory power, with AUC values ranging from 0.554 to 0.842. Sensitivity and specificity varied among the miRNAs and comparisons, with miR-181a consistently demonstrating high sensitivity (> 89%) and moderate to high specificity (> 83.33%) across

Table 2. Salivary MIR-181 Family MicroRNAs as Biomarkers for Periodontitis Severity.

MIR-181 Family MicroRNAs	Periodontitis vs. Healthy controls	Mild to moderate periodontitis vs. Healthy controls	Advanced periodontitis vs. Healthy controls	Advanced vs. Mild to moderate
miR-181a				
AUC	0.854	0.779	0.944	0.999
P	< 0.001	< 0.001	< 0.001	< 0.001
Sensitivity %	98.00	96.34	100.00	98.53
Specificity %	66.67	66.67	88.89	100.00
miR-181b				
AUC	0.660*	0.588*	0.746*	0.600*
P	< 0.001	0.052	< 0.001	0.031
Sensitivity %	56.00	51.22	67.65	85.29
Specificity %	80.00	75.56	80.00	39.02
miR-181c				
AUC	0.747*	0.667	0.845*	0.687*
P	< 0.001	< 0.001	< 0.001	< 0.001
Sensitivity %	65.33	51.22	82.35	79.41
Specificity %	81.11	81.11	81.11	56.10
miR-181d				
AUC	0.602*,&	0.570*	0.642*,&	0.589*
P	0.006	0.111	0.002	0.064
Sensitivity %	26.67	92.68	69.12	26.47
Specificity %	90.00	21.11	55.56	92.68
Combined				
AUC	0.894*,#,@	0.828#,@	0.971*,#,@	1.000#,@
P	< 0.001	< 0.001	< 0.001	< 0.001
Sensitivity %	95.33	92.68	98.53	100.00
Specificity %	68.89	67.78	92.22	100.00

Area Under the Curve (AUC); * indicates the difference between AUC areas compared to miR-181a; # indicates the difference between AUC areas compared to miR-181b; & indicates the difference between AUC areas compared to miR-181c; @ indicates the difference between AUC areas compared to miR-181d.

all comparisons. MiR-181b showed similar trends in sensitivity (> 57.35%) and specificity (> 77.78%), while miR-181c exhibited moderate sensitivity (ranging from 69.51% to 94.12%) and specificity (ranging from 48.78% to 76.67%). MiR-181d displayed varying sensitivity (ranging from 78.05% to 95.59%) and lower specificity (ranging from 24.39% to 71.11%). The combined diagnostic potential of the miR-181 family microRNAs outperformed individual miRNAs, with significantly higher AUC values across all comparisons: 0.974 for periodontitis vs. healthy controls, 0.957 for mild to moderate periodontitis vs. healthy controls, 0.999 for advanced periodontitis vs. healthy controls, and 0.998 for advanced vs. mild to moderate periodontitis (all $P < 0.001$). Sensitivity ranged from 97.07% to 100%, and specificity from 87.78% to 100%.

Statistical analysis using the DeLong test revealed significant differences in AUROC among miR-181 family in various comparisons. Specifically, for periodontitis vs. healthy controls, significant differences were observed for miR-181a vs. miR-181c ($P = 0.004$), miR-181a vs. miR-181d ($P = 0.001$), miR-181b vs. miR-181c ($P = 0.020$), miR-181b *vs.* miR-181d ($P = 0.005$), and miR-181a/miR-181b/ miR-181c/ miR-181d vs. combined (all $P < 0.05$). For mild to moderate periodontitis vs. healthy controls, significant differences were found for miR-181a vs. miR-181c ($P = 0.006$), miR-181a vs. miR-181d ($P = 0.010$), miR-181b vs. miR-181c ($P = 0.006$), miR-181b vs. miR-181d ($P = 0.010$), and miR-181a/miR-181b/ miR-181c/miR-181d alone vs. combined (all $P < 0.05$). For advanced periodontitis vs. healthy controls, significant differences were seen for miR-181a vs. miR-181c ($P = 0.028$), miR-181a vs. miR-181d ($P < 0.001$), miR-181b vs. miR-181d ($P = 0.004$), miR-181c vs. miR-181d ($P < 0.026$), and miR-181b/ miR-181c/ miR-181d vs. combined (all $P < 0.05$). For advanced periodontitis vs. mild to moderate periodontitis, all comparisons showed significant differences ($P < 0.05$).

Correlation Between GCF Inflammatory Biomarkers and miR-181 Family in Periodontitis

Table 4 demonstrates the levels of inflammatory biomarkers (TNF-α, IL-6, and IL-1β) in GCF across various groups: healthy controls, patients with mild to moderate

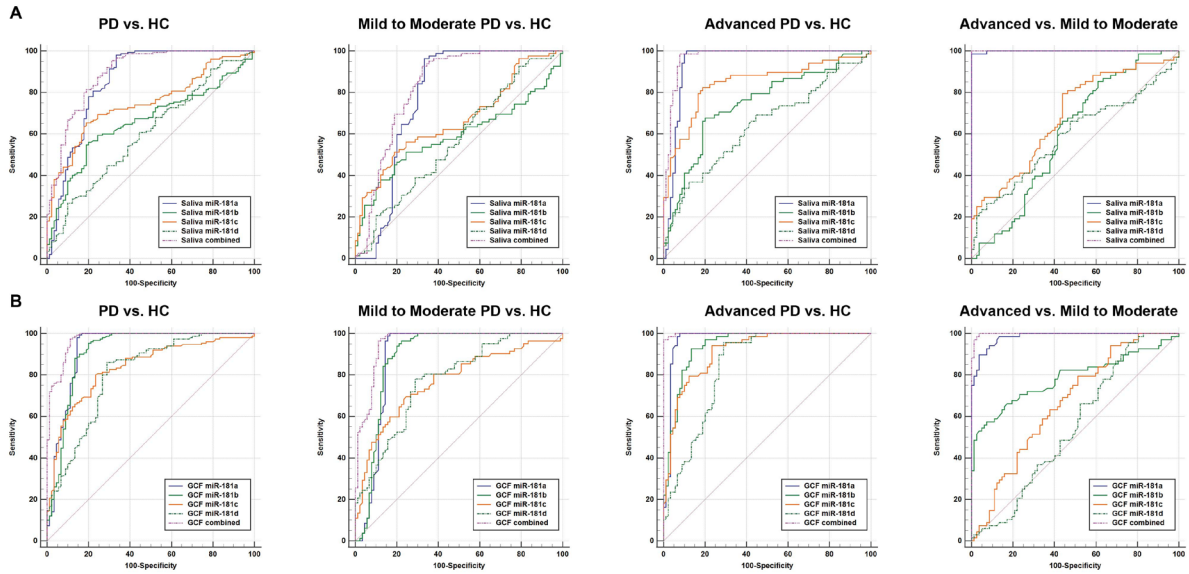

Fig. 2. miR-181 family microRNAs in oral biofluids as biomarkers for periodontitis severity.
A: The analysis of ROC curves demonstrated the discriminatory performance of salivary miRNAs (miR-181a, miR-181b, miR-181c, and miR-181d) in distinguishing periodontitis (PD) patients from healthy controls (HC), as well as across different stages of periodontitis severity. B: The analysis of ROC curves demonstrated the discriminatory performance of miR-181 family members in gingival crevicular fluid (GCF) in distinguishing periodontitis patients from HC, as well as across different stages of periodontitis severity.

periodontitis, and patients with advanced periodontitis. The results indicate significantly elevated levels of these inflammatory biomarkers in both the mild to moderate and advanced periodontitis groups compared to healthy controls (all $P < 0.05$). Moreover, the advanced periodontitis group exhibited further increases in biomarker levels compared to the mild to moderate periodontitis group (all $P < 0.05$). Additionally, Spearman correlation analysis revealed a negative correlation between miR-181 family expression in GCF and the levels of TNF-α (with correlation coefficients for miR-181a, miR-181b, miR-181c, and miR-181d being −0.844, −0.693, −0.608, −0.432, respectively), IL-6 (with correlation coefficients of −0.892, −0.705, −0.566, −0.363, respectively), and IL-1β (with correlation coefficients of −0.407, −0.415, −0.349, −0.380, respectively), all of which were statistically significant (all $P < 0.001$, as depicted in Fig. 3).

Discussion

The miR-181 family, comprising miR-181a/b/c/d, has been extensively studied and implicated in various biological processes, including the regulation of inflammation (Rezaei et al. 2020; Bell-Hensley et al. 2023). For instance, studies have shown significant decreases in miR-181a and miR-181b levels in mouse intestinal epithelial cells during dextran sulfate sodium-induced colitis, and miR-181a downregulation has been observed in patients with ulcerative colitis (Jimenez et al. 2022). Additionally, miR-181 family members have been associated with vascular inflammation (Sun et al. 2014). Moreover, miR-181 family members have been associated with vascular inflammation, with up-regulation observed in epididymal white adipose tissue during diet-induced obesity, contributing to a pro-inflammatory state and insulin resistance (Virtue et al. 2019). MiR-181b plays a role in controlling vascular inflammation in patients with diabetes (Witkowski et al. 2020).

In our study, we aimed to investigate the expression levels of miR-181 family members in saliva and GCF samples collected from individuals with varying degrees of periodontitis severity. The utilization of both GCF and saliva aimed to identify inflammation-specific biomarkers, with GCF providing a reliable indication of periodontal inflammation despite its challenging collection process, while saliva offers a more accessible sampling method, thus facilitating the determination of site-specific markers (Katsiki et al. 2021; Grant et al. 2022). We observed significant differences in the expression levels of miR-181a, miR-181b, and miR-181c among the study groups, indicating an association with periodontitis severity. Interestingly, miR-181d expression was decreased in both mild to moderate and advanced periodontitis groups compared to healthy controls, with significant differences observed only in saliva samples between healthy controls and advanced periodontitis patients. Similarly, previous studies have shown downregulation of miR-181 family members in inflamed human pulp, suggesting their involvement in pulp inflammation (Zhong et al. 2012). MiR-181b and miR-181c also exhibited significant downregulation in apical periodontitis and pulpal inflammation, indicating their potential utility in diagnostic and therapeutic approaches (Al Gashaamy et al. 2023). The observed associations between miR-181 family expression and periodontitis severity suggest their potential

Table 3. MiR-181 Family MicroRNAs in Gingival Crevicular Fluid as Biomarkers for Periodontitis Severity.

MIR-181 Family MicroRNAs	Periodontitis vs. Healthy controls	Mild to Moderate periodontitis vs. Healthy controls	Advanced periodontitis vs. Healthy controls	Advanced vs. Mild to Moderate
miR-181a				
AUC	0.927	0.890	0.972	0.984
P	< 0.001	< 0.001	< 0.001	< 0.001
Sensitivity %	99.33	100.00	100.00	89.71
Specificity %	84.44	83.33	92.22	96.34
miR-181b				
AUC	0.913	0.889	0.941	0.785*
P	< 0.001	< 0.001	< 0.001	< 0.001
Sensitivity %	95.33	96.34	92.65	57.35
Specificity %	80.00	77.78	86.67	92.68
miR-181c				
AUC	0.835*,#	0.769*,#	0.915*	0.667*,#
P	< 0.001	< 0.001	< 0.001	< 0.001
Sensitivity %	80.00	69.51	94.12	79.41
Specificity %	76.67	75.56	76.67	48.78
miR-181d				
AUC	0.806*,#	0.776*,#	0.842*,#,&	0.554*,#,&
P	< 0.001	< 0.001	< 0.001	0.254
Sensitivity %	86.00	78.05	95.59	95.59
Specificity %	71.11	71.11	71.11	24.39
Combined				
AUC	0.974*,#,&,@	0.957*,#,&,@	0.999#,&,@	0.998*,#,&,@
P	< 0.001	< 0.001	< 0.001	< 0.001
Sensitivity %	97.33	97.56	97.07	100.00
Specificity %	88.89	87.78	100.00	96.34

Area Under the Curve (AUC); * indicates the difference between AUC areas compared to miR-181a; # indicates the difference between AUC areas compared to miR-181b; & indicates the difference between AUC areas compared to miR-181c; @ indicates the difference between AUC areas compared to miR-181d.

as non-invasive biomarkers for disease detection and prognosis. Salivary miRNAs, particularly miR-181a, exhibited robust discriminatory ability across various comparisons, with high sensitivity and moderate to high specificity, indicating their potential diagnostic value. MiR-181c also showed promising discriminatory ability, especially in distinguishing advanced periodontitis from healthy controls and between different stages of periodontitis severity. Conversely, miR-181d demonstrated limited discriminatory power in most comparisons. However, the combined diagnostic potential of miR-181 family microRNAs outperformed individual miRNAs, achieving significantly higher Area Under the Curve (AUC) values across all comparisons. These findings suggest the potential utility of miR-181 family microRNAs as non-invasive biomarkers for periodontitis severity assessment. Statistical analysis using the DeLong test revealed significant differences in AUC values among miR-181 family members in various comparisons, highlighting the importance of considering the specific microRNA profiles in periodontitis diagnosis. For instance, miR-181a exhibited significant differences when compared to miR-181b, miR-181c, and miR-181d in various comparisons, emphasizing its distinct discriminatory ability. Similarly, significant differences were observed between miR-181a and miR-181c, miR-181d, and their combinations, indicating variations in their diagnostic performance.

Previous studies have consistently shown that downregulation of miR-181 family members is associated with increased production of pro-inflammatory cytokines, while their overexpression leads to anti-inflammatory responses (Hutchison et al. 2013). For example, miR-181a has been observed to attenuate nasal epithelial cell inflammation and decrease the production of inflammatory cytokines in various inflammatory conditions (Jiang et al. 2018, Long and Zhang 2021). Similarly, overexpression of miR-181b and miR-181 enhanced LPS-induced production of pro-inflammatory cytokines and HMGB1, while overexpression resulted in increased expression of the anti-inflammatory cytokine IL-10 (Hutchison et al. 2013, Yang et al. 2021). Furthermore, the downregulation of miR-181a upon stimulation of human pulp fibroblasts with LPS from

Table 4. Inflammatory Biomarkers in Gingival Crevicular Fluid (GCF) from Periodontitis.

	Healthy controls	Mild to Moderate periodontitis	Advanced periodontitis
TNF-α (pg/mL)	19.95 (17.78-22.53)	24.80 (22.38-27.03)*	32.05 (28.73-35.03)*,#
IL-6 (pg/mL)	68.80 (59.00-78.85)	169.70 (142.40-202.6)*	289.70 (249.40-328.40)*,#
IL-1β (ng/mL)	41.90 (23.98-67.05)	52.30 (28.90-83.75)*	79.45 (41.15-101.90)*,#

Tumor Necrosis Factor-alpha (TNF-α); Interleukin-6 (IL-6); Interleukin-1beta (IL-1β). * indicates significance compared to healthy controls ($P < 0.05$), # indicates significance compared to mild to moderate periodontitis ($P < 0.05$).

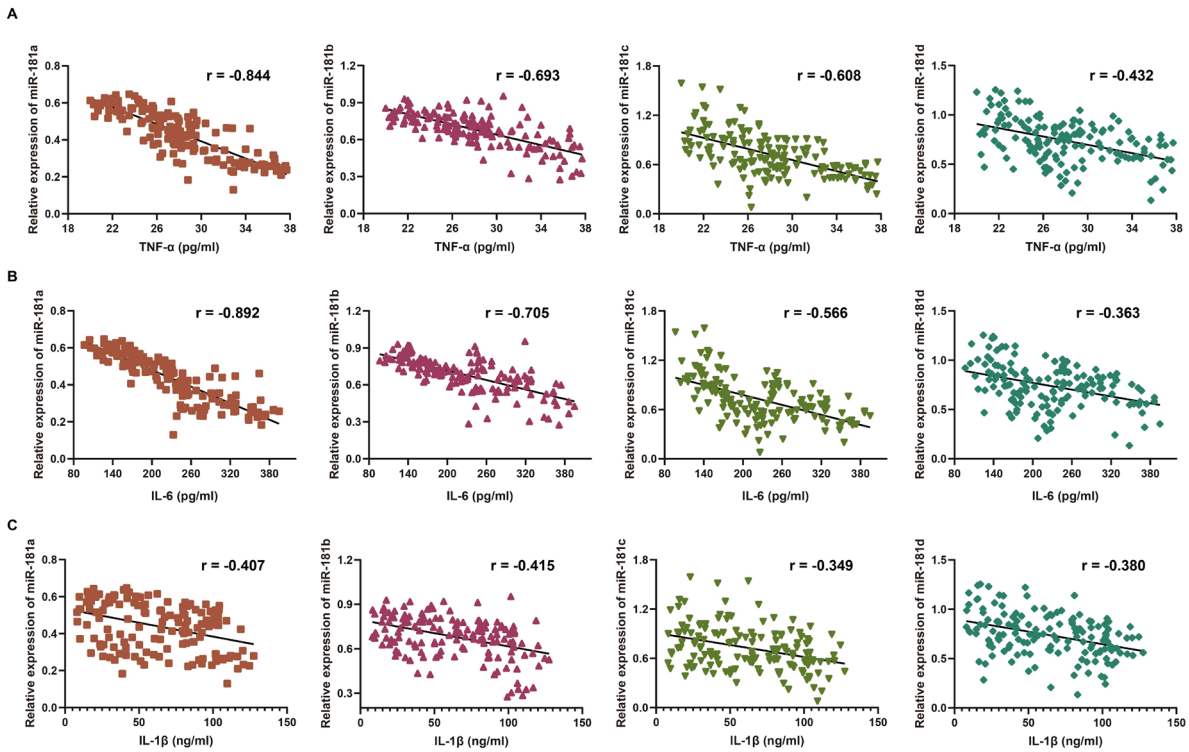

Fig. 3. Correlation between inflammatory biomarkers and miR-181 family expression in gingival crevicular fluid from periodontitis patients
A: Tumor Necrosis Factor-alpha (TNF-α), B: Interleukin-6 (IL-6), C: Interleukin-1beta (IL-1β)

Porphyromonas gingivalis further underscores its involvement in pulp pathology through specific regulatory functions (Galicia et al. 2014). Our Spearman correlation analysis revealed a significant negative correlation between miR-181 family expression in GCF and the levels of TNF-α, IL-6, and IL-1β.

While our study provides valuable insights into the potential diagnostic utility of salivary and GCF miR-181 family microRNAs in periodontitis, several limitations should be acknowledged. Firstly, our investigation did not undertake a comprehensive exploration of the target genes of the miR-181 family. Consequently, the precise mechanistic pathways through which these microRNAs exert their effects remain incompletely understood. Future studies could leverage bioinformatics tools such as TargetScan and miRWalk to delineate the specific targets of miR-181 family members and their roles in periodontal inflammation. Additionally, our focus was primarily on miR-181 family members, neglecting other potential miRNA biomarkers for periodontitis. Moreover, the relatively small sample size necessitates validation in larger cohorts to ensure the robustness of our findings. Future research should further investigate the mechanistic roles of miR-181 family members in periodontal inflammation and explore their potential as therapeutic targets for managing periodontitis.

In conclusion, our study highlights the promising utility of miR-181 family microRNAs in oral biofluids as biomarkers for periodontitis severity. These findings pave the way for further research into non-invasive diagnostic approaches and personalized treatment strategies for periodontal disease, ultimately enhancing patient outcomes and advancing periodontal care.

Authors Contributions

Q.L. and J.-J.Z. contributed equally to this work. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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