The Expression of miR-23a and miR-146a in the Saliva of Patients with Periodontitis and Its Clinical Significance

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Background. This study is aimed at exploring the significance of the expression of miR-23a and miR-146a in patients with periodontitis and their correlations with inflammatory factors. Methods. A total of 120 patients with chronic periodontitis admitted to the department of stomatology in Yantai Yuhuangding Hospital from August 2017 to December 2018 were enrolled as a study group, and 80 healthy volunteers in physical examination during the same period were enrolled as a control group. The expression of miR-23a, miR-146a, interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-17 (IL-17) in the saliva of people in the two groups was determined using the quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). Results. The study group showed significantly higher relative expression of saliva miR-23a and miR-146a than the control group. The area under the curve (AUC) of saliva miR-23a and miR-146a for diagnosing periodontitis was 0.857 and 0.886, respectively. The expression of saliva miR-23a and miR-146a increased with the deterioration of periodontitis in the patients. After basic treatment, the study group showed significantly decreased expression of saliva miR-23a and miR-146a. Patients in the study group showed significantly higher levels of saliva IL-1β, IL-6, and IL-17 than those in the control group, and their saliva miR-23a and miR-146a were positively correlated with their saliva IL-1β, IL-6, and IL-17, respectively. Conclusion. Saliva miR-23a and miR-146a can be used as biomarkers for the diagnosis and assessment of periodontitis, and they may have regulatory relationships with IL-1β, IL-6, and IL-17.

1. Introduction

Periodontitis is a chronic destructive disease of periodontal tissues caused by oral flora disorder [1]. It is a common disease. According to statistics, 10-15% of the global population suffers from severe periodontitis [2]. It not only has negative effects on patients’ oral health, emotion, speech, and nutrition but also increases the risks of systemic diseases such as cardiovascular disease, diabetes, and disease of the respiratory system. It may also affect pregnancy outcomes [3, 4]. Because the symptoms of early periodontitis are not obvious and oral endoscopy is relatively costly and difficult to be widely carried out, the diagnosis level of early periodontitis is relatively low, and most patients have already suffered from tooth loss at the time of diagnosis and are already under serious negative impacts of it on their life quality. Therefore, it is of great clinical value to find easily detectable and economic biomarkers for patients with periodontitis.

MicroRNA (miR) is a short-chain noncoding RNA, with the ability to regulate physiological and pathological processes by blocking protein translation or inhibiting target gene expression through inducing mRNA degradation [5]. Because miR can be involved in cell growth, proliferation, apoptosis, and other biological functions, it has become a research hotspot [6]. miR is a major regulator of inflammation, and the imbalance of molecular processes involved in its induced inflammatory pathway has been proved to conduce to the development of inflammatory diseases [7]. miR-23a and miR-146a, major members of miR, are abnormally expressed in many inflammatory diseases. For example, a study by Hu et al. revealed that miR-23a showed...
downregulated expression in articular cartilage tissues of patients with rheumatoid arthritis, and it could inhibit the interleukin-17 (IL-17-) mediated expression of proinflammatory mediators by targeting IKKα [8], and a study by Wade et al. pointed out that miR-23a decreased in synovial tissues of patients with psoriatic arthritis, and its decreased expression can induce activation of synovial fibroblasts [9]. A study by Zeng et al. concluded that upregulation of miR-146a expression was helpful to inhibit inflammatory responses in rats with acute lung injury induced by lipopolysaccharide [10]. In addition, a study by Zhou et al. revealed that in terms of rheumatoid arthritis, miR-146a promoted the proinflammatory phenotypes that regulate T cells by intensifying the activation of STAT1, thus contributing to the pathogenesis of rheumatoid arthritis [11]. All the above studies revealed that miR-23a and miR-146a were closely related to inflammatory diseases. However, there are few reports on miR-23a and miR-146a in periodontitis. Therefore, we selected miR-23a and miR-146a as research factors for this study.

This study explored the expression of saliva miR-23a and miR-146a in patients and healthy people and the correlations of the two factors with inflammatory factors so as to find an easily detectable and economical biomarker for the diagnosis and prognosis of periodontitis.

2. Materials and Methods

2.1. Patients. A total of 120 patients with chronic periodontitis admitted to the department of stomatology in Yantai Yuhuangding Hospital from August 2017 to December 2018 were enrolled as a study group. The inclusion criteria were as follows: patients meeting the diagnostic criteria of periodontitis [12], patients with detailed clinical data, patients with more than 16 remaining teeth in their oral cavities, patients who had not taken any antibiotics or immunoregulation drugs within 6 months before participating in this study and had not received any drugs for periodontitis or surgical treatment for it within 6 months before it, and patients with normal communication ability. In addition, 80 healthy volunteers undergoing physical examination in Yantai Yuhuangding Hospital at the same time were selected as a control group. The exclusion criteria were as follows: patients with pneumonia, osteoarthritis, or other inflammatory diseases; patients with infectious diseases or tumor diseases; women in pregnancy, lactation, or menstruation; or patients who had received orthodontic treatment. This study was approved by the Medical Ethics Committee of Yantai Yuhuangding Hospital, and each person in the two groups signed an informed consent form after understanding the study.

2.2. Main Instruments and Reagents. The materials used in this study include the following: TRIzol extraction kit (Wuhan Chundu Biotechnology Co., Ltd., China, CDL-G-4396); reverse transcription kit (Tiangen Biotech (Beijing) Co., Ltd., China, FP209); real-time fluorescence ratio PCR instrument (ABI Companies, United States, 7300); spectrophotometer (Hach Company, United States, DR5000); enzyme-linked immunosorbent assay (ELISA) kits for human interleukin-1β (IL-1β), human interleukin-6 (IL-6), and human interleukin-17 (IL-17) (Elabscience, China, E-EL-H0149C, E-EL-H102C, and E-EL-H105km-1); and multifunction enzyme marking instrument (Shanghai Flash Spectrum Biotechnology Co., Ltd., China, SuPerMax 3100). All primers were designed and synthesized by Takara Bioengineering Co., Ltd. (China). See Table 1.

2.3. Severity Grading. The severity of periodontitis was evaluated according to the periodontal probing depth (PD), gingival index (GI), attachment loss (AL), and X-ray film as follows: mild periodontitis: GI > 1, AL: 1-2 mm, alveolar bone resorption length < 1/3 root length, and PD ≤ 4 mm; moderate periodontitis: GI > 1, AL: 3-4 mm, alveolar bone resorption length: 1/3-1/2 root length, and PD ≥ 6 mm; severe periodontitis: GI > 1, AL ≥ 5 mm, alveolar bone resorption length ≥ 1/2 root length, and PD > 6 mm.

2.4. Basic Periodontal Treatment and Reexamination. Before treatment, patients in the study group were given guidance about oral hygiene and diet before being treated with oral prophylaxis, gingival flap operation, or others. At 21 d after treatment, they were reexamined.

2.5. Specimen Collection. The swab test on premises was employed to collect saliva specimens from people in the two groups as follows [13]. They were prohibited from eating and overexercising 2 h before specimen collection. Their oral cavities were wiped with cotton swab stained with 2% citric acid to stimulate saliva secretion, and their saliva was collected with RNA enzyme-free centrifuge tubes, centrifuged at 3000 r/min for 15 min at 4°C, and the upper layer of them was taken and stored in a -80°C refrigerator.

2.6. Determination of the Expression of miR-23a and miR-146a (qRT-PCR). The total RNA was extracted with a TRIzol extraction kit, and the concentration and purity of it were determined using a DR5000 ultraviolet spectrophotometer. Reverse transcription of it was carried out using the reverse transcription kit in strict accordance with the instructions of the kit. Subsequently, a polymerase chain reaction (PCR) amplification experiment was performed, with U6 as the internal reference. The PCR reaction system consisted

| Group | Upstream (5’-3’) | Downstream (5’-3’) |
|-------|------------------|--------------------|
| miR-23a | GGGGATCACCATTGGCCAGG | AGTGGCGTGTCGTGGAGTC |
| miR-146a | TGTTCTGAAGAGCCTCCTGTAAC | TCCTCTGATGATGTCGAATGTAC |
| U6 | CTTGGCGACACATATAAAT | CGCTTCACGAATTGTGGTCAT |

Table 1: Primer sequences.
of 20 μl of the total volume containing 10 μl of 2x Talent qPCR PreMix, 1.25 μl of upstream and downstream primers, respectively, 100 ng of cDNA, and water to adjust the volume. PCR was carried out under predenaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing and extension at 60°C for 15 s. Data in this study were analyzed using 2^-ΔΔct [14].

2.7. Determination of the Expression of Inflammatory Factors (ELISA). ELISA was employed to determine the expression levels of three inflammatory factors in the saliva of the two groups, namely, IL-1β, IL-6, and IL-17, in strict accordance with instructions of ELISA kits for IL-1β, IL-6, and IL-17. A blank well, a standard well, and a well for specimens to be determined were set. A total of 50 μl of standards with different concentrations were added into the standard well, and 10 μl of specimens to be determined and 40 μl of diluent were added into the well for specimens to be determined successively. Then, 100 μl of the horseradish peroxidase-labeled detection antibody was added into the standard well and the well for specimens to be determined, respectively. Each well was blocked with a microplate sealer and incubated in a 37°C incubator for 1 h. After incubation, the microplate sealer was removed. The liquid in each well was

| Group                  | Control group (n = 80) | Study group (n = 120) | χ²/t       | P value |
|------------------------|------------------------|-----------------------|------------|---------|
| Sex                    |                        |                       |            |         |
| Male                   | 49 (61.25)             | 70 (58.33)            |            |         |
| Female                 | 31 (38.75)             | 50 (41.67)            |            |         |
| Age (years)            | 48.34 ± 3.54           | 47.72 ± 4.98          |            |         |
| Weight (kg)            | 63.24 ± 8.33           | 65.56 ± 9.78          |            |         |
| Educational level      |                        |                       |            |         |
| <Senior high school    | 33 (41.25)             | 61 (50.83)            |            |         |
| ≥Senior high school    | 47 (58.75)             | 59 (49.17)            |            |         |
| Dietary favor          |                        |                       |            |         |
| Light                  | 51 (63.75)             | 70 (58.33)            |            |         |
| Heavy                  | 29 (36.25)             | 50 (41.67)            |            |         |
| Place of residence     |                        |                       |            |         |
| Urban area             | 34 (42.50)             | 61 (50.83)            |            |         |
| Rural area             | 46 (57.50)             | 59 (49.17)            |            |         |
| Having exercise habit or not |                  |                       |            |         |
| Yes                    | 37 (46.25)             | 49 (40.83)            |            |         |
| None                   | 43 (53.75)             | 71 (59.17)            |            |         |
| Marital status         |                        |                       |            |         |
| Married                | 74 (92.50)             | 106 (88.33)           |            |         |
| Unmarried              | 5 (6.25)               | 9 (7.50)              |            |         |
| Divorced               | 1 (1.25)               | 5 (4.17)              |            |         |
| Smoking history        |                        |                       |            |         |
| Yes                    | 45 (56.25)             | 79 (65.83)            |            |         |
| None                   | 35 (43.75)             | 41 (34.17)            |            |         |
| Drinking history       |                        |                       |            |         |
| Yes                    | 48 (60.00)             | 69 (57.50)            |            |         |
| None                   | 32 (40.00)             | 51 (42.50)            |            |         |

![Figure 1: Comparison between the two groups in the expression of saliva miR-23a and miR-146a. The study group showed significantly higher relative expression of saliva miR-23a and miR-146a than the control group (all P < 0.05). Note: * indicates that in comparison with the control group, P < 0.05.](image-url)
discarded, and each well was washed with washing solution for 1 min and patted to dry. This step was repeated 5 times. Each well was added with 50 μl of chromogenic reagent A and 50 μl of chromogenic reagent B, respectively, and the liquid in each well was mixed well. Subsequently, each well was developed in the dark at 37 °C for 10-15 min and then added with 50 μl of sulfuric acid to terminate the reaction. The optical density of each well was detected using an enzyme marking instrument at 450 nm, on which the levels of IL-1β, IL-6, and IL-17 were calculated.

2.8. Statistical Analysis. In this study, the data were statistically analyzed using SPSS 21.0 (EASY BIO Company) and visualized to figures using GraphPad Prism 7. Enumeration data were expressed by the number of cases/percentage (n (%)), and comparison between the two groups in terms of them was performed using the chi-squared test. Measurement data were expressed by the mean ± standard deviation (x ± sd), and comparison between the two groups in terms of them was performed using the independent-samples t-test. Comparison of one same group before and after treatment was performed using the paired t-test. Receiver operating characteristic (ROC) curves were drawn to evaluate the diagnostic value of saliva miR-23a and miR-146a in diagnosing periodontitis, and correlation analysis was performed using the Pearson correlation method. P < 0.05 indicated a significant difference.

3. Results

3.1. Comparison between the Two Groups in General Data. There were no significant differences between the two groups in general data including sex, age, weight, educational level, dietary favor, place of residence, exercise habit, marital status, smoking history, and drinking history (all P > 0.05). See Table 2.

3.2. Comparison between the Two Groups in the Expression of Saliva miR-23a and miR-146a. The relative expression of saliva miR-23a and miR-146a in the control group was 1.02 ± 0.12 and 1.06 ± 0.15, respectively, while that in the study group was 1.89 ± 0.49 and 2.12 ± 0.56, respectively, so it was apparent that the study group showed significantly higher relative expression of saliva miR-23a and miR-146a than the control group (both P < 0.05). See Figure 1.

3.3. Diagnostic Value of Saliva miR-23a and miR-146a for Periodontitis. We drew receiver operating characteristic (ROC) curves of saliva miR-23a and miR-146a for diagnosing periodontitis, finding that the area under the curve (AUC) of saliva miR-23a for diagnosing periodontitis was 0.857 (95% CI: 0.802-0.912), and its cutoff value, diagnostic sensitivity, and diagnostic specificity were 1.429, 81.67%, and 83.75%, respectively. It was also revealed that the AUC of saliva miR-146a for diagnosing periodontitis was 0.886 (95% CI: 0.838-0.934), and its cutoff value, diagnostic sensitivity, and diagnostic specificity were 1.589, 82.50%, and 86.25%, respectively. See Table 3 and Figure 2.

3.4. Changes of the Expression of miR-23a and miR-146a in Patients under Different Severities. The relative expression of saliva miR-23a in patients with mild periodontitis, patients with moderate periodontitis, and patients with severe periodontitis was 1.69 ± 0.08, 1.88 ± 0.12, and 2.04 ± 0.10, while the relative expression of saliva miR-146a in them was 1.87 ± 0.09, 2.08 ± 0.11, and 2.34 ± 0.13, respectively, so it was apparent that the expression of miR-23a and miR-146a increased with the deterioration of periodontitis, and the two groups had significant differences in the expression of them (both P < 0.05). See Figure 3.

3.5. Changes of miR-23a and miR-146a in the Study Group before and after Treatment. The expression of saliva miR-23a and miR-146a in the study group was significantly lower than that after treatment (1.45 ± 0.33 vs. 1.89 ± 0.49; 1.59 ± 0.38 vs. 2.12 ± 0.56) (both P < 0.05). See Figure 4.

3.6. Comparison between the Two Groups in the Expression of Saliva IL-1β, IL-6, and IL-17. The levels of saliva IL-1β, IL-6, and IL-17 in the control group were 1.88 ± 0.63, 3.24 ± 0.85, and 7.57 ± 1.58 ng/l, respectively, and those in the study group were 3.35 ± 0.94, 6.14 ± 1.32, and 12.57 ± 2.68 ng/l, respectively, so it was apparent that the study group showed significantly higher levels of IL-1β, IL-6, and IL-17 than the control group (all P < 0.05). See Figure 5.

| Group       | AUC   | 95% CI     | Standard error | Cutoff value | Sensitivity (%) | Specificity (%) |
|-------------|-------|------------|----------------|--------------|----------------|----------------|
| miR-23a     | 0.857 | 0.802-0.912| 0.028          | 1.429        | 81.67          | 83.75          |
| miR-146a    | 0.886 | 0.838-0.934| 0.025          | 1.589        | 82.50          | 86.25          |

Figure 2: ROC curves of saliva miR-23a and miR-146a for diagnosing periodontitis. The AUCs of saliva miR-23a and miR-146a were 0.857 and 0.886, respectively. Their diagnostic sensitivities were 81.67% and 82.50%, respectively, and their diagnostic specificities were 83.75% and 86.25%, respectively.
3.7. Analysis of the Correlation of miR-23a and miR-146a with IL-1β, IL-6, and IL-17. Pearson correlation analysis revealed that in the study group, saliva miR-23a was obviously and positively correlated with saliva IL-1β, IL-6, and IL-17, respectively (r = 0.542, P < 0.001; r = 0.638, P < 0.001; and r = 0.606, P < 0.001). Saliva miR-146a was also obviously and positively correlated with them, respectively (r = 0.606, P < 0.001; r = 0.639, P < 0.001; and r = 0.646, P < 0.001). See Figure 6.

4. Discussion

Early diagnosis and observation of periodontitis can provide important information for periodontitis treatment and facilitate the selection of an appropriate treatment plan, so they are a key step to alleviate patients’ symptoms and improve their life quality [15]. At present, the diagnosis level for early periodontitis is unsatisfactory. Although imaging evaluation can provide information about the severity of periodontitis based on its results in terms of exploration depth, attachment level, bleeding detection, and loss of alveolar bone, it is relatively costly and unable to detect disease activity, resulting in certain limitations [16]. Therefore, it is of great significance to find biological factors closely related to the diagnosis and assessment of periodontitis.

Saliva is a biological fluid with abundant clinical information, which is easy to collect and store, so it is an ideal choice for the early detection of diseases [17]. At present,
saliva miR is proved to be feasible as a disease diagnosis method [18]. A study showed that saliva miR-21 could be used as a biomarker for diagnosing colorectal cancer, and its sensitivity was higher than that of serum miR-21 (65% vs. 97%) [19]. One other study concluded that miR-1246 and miR-4644 in the saliva exosome could be used as biomarkers of identifying patients with pancreatic cancer or cholangiocarcinoma [20]. In addition, a study by Humeau et al. revealed that miR-23a showed significantly upregulated expression in the saliva of patients with pancreatic cancer, and its sensitivity and specificity for diagnosing pancreatic cancer were 85.7% and 100%, respectively [21]. We determined the expression of miR-23a and miR-146a in the saliva of patients with periodontitis and healthy volunteers, finding that patients in the study group showed significantly higher expression of saliva miR-23a and miR-146a than healthy people in the control group. It indicated that saliva miR-23a and miR-146a may be used as biomarkers for periodontitis diagnosis. To verify it, we drew ROC curves of saliva miR-23a and miR-146a for periodontitis diagnosis, which showed that the AUCs of saliva miR-23a and miR-146a for periodontitis diagnosis were 0.857 and 0.886, respectively; their diagnostic sensitivities were 81.67% and 82.50%, respectively, and their diagnostic specificities were 83.75% and 86.25%, respectively. It suggested that saliva miR-23a and miR-146a had certain diagnostic value for periodontitis. We also determined the expression of miR-23a and miR-146a in the saliva of patients with periodontitis at different stages and patients in the study group, finding that the expression of saliva miR-23a and miR-146a increased with the deterioration of the disease, and patients in the study group showed significantly decreased expression of saliva miR-23a and miR-146a after treatment. It indicated that saliva miR-23a and miR-146a were expected to be biomarkers of periodontitis.

Periodontitis is one of the most common infection-driven inflammatory diseases characterized by gingival inflammation and bone loss [22]. The occurrence of periodontitis may lead to increases in local proinflammatory cytokines and mediators and thus cause specific local host responses [23]. IL-1β, IL-6, and IL-17 are three common inflammatory factors involved in the occurrence and development of various inflammatory diseases including periodontitis. Fine et al. longitudinally evaluated periodontal disease progression in children at risk for aggressive periodontitis and reported that IL-1β demonstrated high specificity and sensitivity to predict alveolar bone loss [24]. IL-1β stimulated endothelial cells to induce selectins, which facilitate the recruitment of leukocytes, activate macrophage IL-1 production, and stimulate the production of inflammatory mediators. A study pointed out that IL-1β and IL-6 were highly expressed in the saliva of patients with periodontitis and had diagnostic value for periodontitis [25]. A previous systematic review and meta-analysis indicated that macrophage inflammatory protein-1α had excellent diagnostic accuracy while IL-1β and IL-6 had acceptable diagnostic values [26]. One other study revealed that IL-6 and IL-17 were highly expressed in the saliva of patients with periodontitis, and they increased with the deterioration of periodontitis in the patients [27]. Ozçaka et al. state that there is an emerging understanding of the role of T helper 17 and IL-17 cytokines in periodontal diseases, and little is known about their main role in disease pathogenesis and host conservation [28]. Pradeep et al. reported in their study that the GCF concentration of IL-17 is near to zero. Since the results of their research indicated a lack of IL-17 in GCF, they recognized that it cannot be considered a biomarker in periodontal disease development [29]. Isaza-Guzmán et al. also found no relationship between IL-17 salivary concentrations and chronic periodontitis and stated

**Figure 6:** Analysis of the correlation of saliva miR-23a and miR-146a with IL-1β, IL-6, and IL-17. Pearson correlation analysis revealed that in the study group, saliva miR-23a was obviously and positively correlated with saliva IL-1β, IL-6, and IL-17, respectively ($r = 0.542$, $P < 0.001$; $r = 0.638$, $P < 0.001$; and $r = 0.606$, $P < 0.001$) (a–c), and saliva miR-146a was also obviously and positively correlated with them, respectively ($r = 0.606$, $P < 0.001$; $r = 0.639$, $P < 0.001$; and $r = 0.646$, $P < 0.001$) (d–f).
that it is futile to decide on the role of this cytokine in periodontal disease or its severity detection [30]. Our study also revealed that the expression of IL-1β, IL-6, and IL-17 was high in the saliva of the patients with periodontitis, which was similar to the above research results. In addition, we also used the Pearson correlation method to analyze the correlation of saliva miR-23a and miR-146a with saliva IL-1β, IL-6, and IL-17, finding that saliva miR-23a and miR-146a were obviously and positively correlated with saliva IL-1β, IL-6, and IL-17, which suggested that miR-23a and miR-146a had regulatory relationships with IL-1β, IL-6, and IL-17.

Although this study has confirmed that saliva miR-23a and miR-146a can be used as biomarkers for the diagnosis and assessment of periodontitis, it still has some deficiencies. For example, firstly, there were no basic experiments, so the correlations of miR-23a and miR-146a with periodontitis and inflammatory factors were not explored deeply. Secondly, the subjects in the study group and control group were from the same area, resulting in certain limitations in the results. In future research, we will add more experiments to address these deficiencies.

5. Conclusion
To sum up, saliva miR-23a and miR-146a can be used as biomarkers for the diagnosis and assessment of periodontitis, and they may have regulatory relationships with IL-1β, IL-6, and IL-17.

Data Availability
The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
Lanhua Kang and Ning Li performed the experiments, analyzed the data, and wrote the manuscript. Lexiu Wang designed the study. All the authors agreed to be accountable for the accuracy and integrity of all aspects of the research. Lanhua Kang and Lexiu Wang contributed equally to this work as co-first authors.

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