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Assessment of microRNA-146a in generalized aggressive periodontitis and its association with disease severity

S. Ghotloo[1] | H. Motedayyen[1,2] | D. Amani[3] | M. Saffari[4] | M. Sattari[3]

[1]Department of Laboratory Medicine, Kashan University of Medical Sciences, Kashan, Iran

[2]Department of Immunology, Isfahan University of Medical Sciences, Isfahan, Iran

[3]Department of Immunology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[4]Department of Microbiology and Laboratory Medicine, Kashan University of Medical Sciences, Kashan, Iran

Correspondence
Mandana Sattari, Department of Immunology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
Email: mandana.sattari@sbmu.ac.ir

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Background and Objective: MicroRNA-146a (miR-146a) is a small noncoding RNA that plays a critical role in the negative regulation of the innate immune response, and the dysregulation of miR-146a has been associated with several inflammatory disorders. In generalized aggressive periodontitis (GAgP) the degree of clinical inflammation appears to be similar to that of chronic periodontitis, and, in this situation, age of onset and family history are important additional criteria for diagnosis. This study was performed to evaluate the level of miR-146a expressed in gingival tissues of patients with GAgP and its association with disease severity.

Material and Methods: Gingival samples from 18 patients with GAgP and 10 healthy subjects were collected and the level of miR-146a and its targets, including necrosis factor-alpha, interleukin-1beta, and interleukin-6, were assessed using real-time PCR.

Clinical parameters, including probing depth and clinical attachment loss, were measured and their correlations with the level of miR-146a were determined.

Results: Our results demonstrated an elevation in the level of miR-146a expressed in patients with GAgP compared with healthy controls ($P < .001$), which was directly associated with disease severity ($P < .05$). Overexpression of miR-146a was accompanied by a reduction in the levels of pro-inflammatory cytokines.

Conclusions: Our findings suggest that there is an association between miR-146a and GAgP and imply that miR-146a may serve as an indicator of periodontal disease severity. However, further studies and additional information are required to confirm this relationship and the precise role of miR-146a in the development and/or progression of periodontitis.

KEYWORDS

aggressive periodontitis, microRNA-146a, pro-inflammatory cytokines

1 | INTRODUCTION

Periodontitis is a set of chronic inflammatory disorders of tissues surrounding the teeth that leads to pocket formation and breakdown of alveolar bone around the teeth, resulting in tooth loss.[1] Aggressive periodontitis is a less common, but more severe, form of the disease than chronic periodontitis that usually affects younger patients and typically manifests as rapid loss of periodontal attachment and bone destruction.[2,3] The disease is a consequence of accumulation of bacteria in dental plaque.[3,4] Bacterial components, such as lipopolysaccharide (LPS) recognized by toll-like receptors of resident cells and inflammatory leukocytes trigger an intracellular signaling cascade resulting in the activation of nuclear factor-kappa B, a key transcription factor responsible for the expression of many genes involved in inflammation, such as pro-inflammatory cytokines, chemokines, adhesion molecules, and prostaglandins.[5-7] Consequently, leukocytes migrating to the periodontal tissues disrupt homeostatic conditions of these tissues through production of toxic products

(such as reactive oxygen and nitrogen species) and enzymes (such as matrix metalloproteinases), leading to the destruction of soft tissues.[8,9] In addition, alveolar bone loss, a major feature of the disease, occurs because of an imbalance of receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin levels.[10,11] Elevation of RANKL and reduction of osteoprotegerin shift the balance of osteoclastogenesis/osteoblastogenesis to bone resorption.[10,11]

MicroRNAs (miRNAs) were discovered by Ambros in 1993.[12] They are small noncoding RNAs (about 22 nucleotides in length) that contribute to gene regulation by binding to target sites of messenger ribonucleic acid (mRNA).[13] This leads to mRNA degradation or inhibition of protein translation.[13] It appears that > 30% of human genes have complementary sites to the seed region of miRNAs (nucleotides 2-7).[14] Therefore, a large number of genes are regulated by miRNAs. In addition to physiological involvement of miRNAs in a wide range of biological processes, dysregulation of miRNAs has been addressed in a number of diseases, including cancers, and heart, kidney, and neurological diseases.[15-18]

The miR-146 family consists of two evolutionarily conserved members, namely miR-146a and miR-146b, which are located on different chromosomes and for which the mature forms differ by 2 nucleotides.[19] In spite of significant structural similarities between miR-146a and miR-146b, it does not seem that their biological activities are the same.[19] It has been shown that upon LPS stimulation, the mature form of miR-146a is produced in a nuclear factor-kappa B-dependent manner, leading to negative regulation of the innate immune response via the downregulation of pro-inflammatory cytokines, chemokines, and other inflammatory mediators.[19,20] miR-146a is a key regulator of LPS tolerance.[21] LPS tolerance is a phenomenon in which, after prolonged exposure to LPS, monocytes develop a hyporesponsiveness state to subsequent LPS challenge, leading to the control of intensity and duration of inflammation.[21] Therefore, we hypothesized that downregulation of miR-146a or its impaired function may be associated with diseases, such as aggressive periodontitis, in which sustained or exaggerated inflammation contribute to pathogenesis. Herein, we assessed the expression level and functional status of miR-146a in generalized aggressive periodontitis (GAgP) to clarify whether there is any association between miR-146a and this form of periodontitis.

2 | MATERIAL AND METHODS

2.1 | Tissue samples and clinical examination

Periodontists collected periodontal samples from 18 patients with GAgP (7 male and 11 female subjects, mean age = 27 years) during surgical therapy. According to the update of the 1999 American academy of periodontology classification criteria for periodontal disease,[22-24] patients with GAgP are diagnosed based on significant and rapid attachment loss and bone destruction in the presence of low local factors (plaque and calculus) and are usually younger than 30 years of age. Except for the periodontits, the patients were systemically healthy. In addition, they had generalized interproximal attachment loss affecting at least 3 permanent teeth other than the first molars and incisors. In these patients, >30% of sites involved had clinical attachment loss of ≥5 mm and radiographic bone loss of >30%, usually presenting as a vertical bone lesion, as well as the presence of bleeding on probing (BOP). All clinical parameters were measured using a calibrated Michigan O probe with Williams Markings (Hu Friedy, Chicago, IL, USA). Six sites (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) were examined for each tooth. For this group, Phase I periodontal therapy consisted of supra- and subgingival scaling; polishing and oral hygiene instruction was accomplished at least 1 month before surgery; and periodontal charting and BOP evaluation were performed at the first visit and 1 week before surgery to confirm BOP positivity of the site. Exclusion criteria were: any known systemic disease; pregnancy and breast feeding; any history of cigarette smoking, alcohol consumption or drug abuse; any history of medication uptake (such as calcium channel blockers, or anticonvulsive and immunosuppressive agents) that could affect periodontal conditions; any history of allergy; orthodontic therapy; or antibiotic therapy in the preceding 6 months. In addition, sampling was not performed around wisdom teeth, teeth with heavy occlusal interferences, teeth with combined periodontal-endodontal problems, or teeth with pericoronitis and pathologies such as ulcers, cysts, abscesses, or tumors.

Tissue samples of 10 healthy subjects (1 male and 9 female subjects, mean age = 32 years) were obtained during crown-lengthening surgery with the inclusion criteria probing depth <3 mm, clinical attachment loss <3 mm without any radiographic evidence of alveolar bone loss, and no sign of BOP in the operation site at the first visit and at the operation session.

At the first visit, all the local irritating factors, including calculus, bacterial plaque, caries, and unfavorable temporary crowns with subgingival margins, were removed and individuals were instructed to brush their teeth 3 times a day and to floss once daily, and surgery was scheduled for at least 1 week later.

Subjects were included in the study after providing informed consent, in accordance with the Ethics Committee of Shahid Beheshi University of Medical Sciences, in accordance with the Helsinki Declaration of 1975, as revised in 2013.

According to the SD values observed in similar research[1,25,26], sample sizes for the groups of patients and healthy controls were estimated using the following statistical formula:

$$n = \frac{(Z\alpha + Z\beta)^2 \times (S1^2 + S2^2)}{d}$$

α (study accuracy) = % 95; β (study power) = % 80; *d* (effect size) = % 80; $Z_\alpha = 1.96$; $Z_\beta = 0.84$; $S_1 = 1.2$; $S_2 = 1$.

2.2 | Measurement of miR-146a and pro-inflammatory cytokine levels

Total ribonucleic acid (RNA) were isolated from periodontal tissues that were stored in RNAlater (Qiagen, Germantown, MD, USA) at −20°C (mirVana miRNA isolation kit; Ambion, Austin, TX, USA) according to

TABLE 1 Sequences of primers for real-time PCR

Gene	Forward primer (5′-3′)	Reverse primer(5′-3′)
TNFα	GCTGCACTTTGGAGTGATCG	GGGTTTGCTACAACATGGGC
IL1β	GCACGATGCACCTGTACGAT	TGGAGAACACCACTTGTTGC
IL6	CTTCTCCACAAGCGCCTTCG	CTGAGATGCCGTCGAGGATG
GAPDH	CTCTGGTAAAGTGGATATTG	GGTGGAATCATATTGGAACA

Primer sequences for amplification of tumor necrosis factor-alpha *(TNF-α)*, interleukin-1beta (*IL-1β*), interleukin-6 (*IL-6*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs in SYBR Green-based real-time PCR.

the manufacturer's protocol. Tissue samples were homogenized (using 1.0-mm silicon carbide beads; BioSpec products, Bartlesville, OK, USA) before RNA isolation. RNA yield was determined and the purity was assessed using a spectrophotometer (NanoDrop 8000 spectrophotometer; Thermo Fisher Scientific, Wilmington, DE, USA).

For miR-146a, complementary deoxyribonucleic acid (cDNA) synthesis was performed by the TaqMan microRNA reverse transcription kit (Ambion). Afterwards, real-time polymerase chian reaction (PCR) was carried out using TaqMan® Universal Master Mix II, no UNG, and hsa-miRNA146a kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Total RNAs were reverse transcribed to determine the levels of pro-inflammatory cytokine genes expressed, including tumor necrosis factor alpha *(TNF-α)*, interleukin-1β *(IL-1β)*, and interleukin-6 *(IL-6)* (RevertAid First Strand cDNA Synthesis Kit; Thermo Fisher Scientific) according to the manufacturer's protocol.

Real-time PCR was performed on a Rotor Gene 6000 (Qiagen, Hilden, Germany) in a 15-μL reaction mixture containing 7.5 μL of Master Mix (SYBR® Premix Ex Taq™ II; TaKaRa, Kyoto, Japan), 0.5 μL of each 10 pM primer, 0.5 μL of cDNA template, and DNase- and RNase-free water (6 μL). Each reaction was incubated at 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The melting curve analysis was performed in the temperature range from 65°C to 95°C at the end of each run. Sequences of primers are summarized in Table 1.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the expression level of each sample (Table 1). The reaction mixtures and cycling parameters for GAPDH were the same as those used for the cytokines, with the exception of the annealing temperature (55°C for 30 seconds). Relative expression of miR-146a and cytokines were calculated using the $2^{-\Delta\Delta ct}$ method.[27]

2.3 | Statistical analysis

Data were analyzed using SPSS software (v.17; SPSS, Chicago, IL, USA). The Student's *t* test was used to compare gene expression between groups with a normal distribution and the Mann-Whitney test was used to compare gene expression between groups with a non-normal distribution. The correlation study was performed using Pearson's test (in the case of normal distribution) or Spearman's test (in the case of non-normal distribution). A value of $P \leq .05$ was considered statistically significant.

3 | RESULTS

3.1 | Clinical findings

In order to evaluate disease severity, clinical attachment loss and probing depth were measured at disease sites of each study participant. The diseased sites of patients with GAgP demonstrated statistically significantly higher probing depth and clinical attachment loss values when compared with the sample sites of patients in the control group. Despite higher values of both clinical attachment loss and probing depth in patients with GAgP, there was no correlation between clinical attachment loss and probing depth in these patients. As analysis using the Kolmogorov-Smirnov test was unable to identify any normal distribution of data for either clinical attachment loss ($P < .03$) or probing depth ($P < .05$), this might explain the lack of correlation between clinical attachment loss and probing depth.

The average age of patients was significantly lower than those of healthy subjects, suggesting that the younger population has an increased risk for aggressive periodontitis. A summary of clinical and demographic parameters is given in Table 2.

3.2 | Increased expression of miR-146a in GAgP and its association with disease severity

Considering the role of miR-146a as a negative-feedback regulator of inflammation, we investigated whether any change in the level of miR-146a existed in GAgP. We used real-time PCR to measure the miR-146a level in diseased sites of gingival tissues. We found a 17.8-fold elevation in miR-146a expression in patients compared with healthy subjects ($P < .001$) (Figure 1).

To determine the association between the miR-146a level and disease severity, we performed correlation tests. Patients with GAgP who had a higher probing depth score had much more elevated level of miR-146a expression than those patients with lower probing depth (Spearman's correlation coefficient = 0.504, $P < .05$) (Figure 2). However, no significant association between the miR-146a level and clinical attachment loss was observed ($P \approx .17$).

3.3 | Decreased expression of TNF-α, IL-1β, and IL-6 in disease sites in line with the elevated level of miR-146a

To assess the regulatory role of miR-146a, we monitored its effect on the level of pro-inflammatory cytokines expressed. In spite of

TABLE 2 Clinical and demographic findings

Groups	Probing depth (mm)	Clinical attachment loss (mm)	Age (years)	Gender
Aggressive periodontitis	5.61 ± 1.61	7.10 ± 1.9	27 ± 13	11 female subjects 7 male subjects
Healthy subjects	2.00 ± 0.45	2.10 ± 0.54	32 ± 12	9 female subjects 1 male subject

Data are shown as mean ± SD.

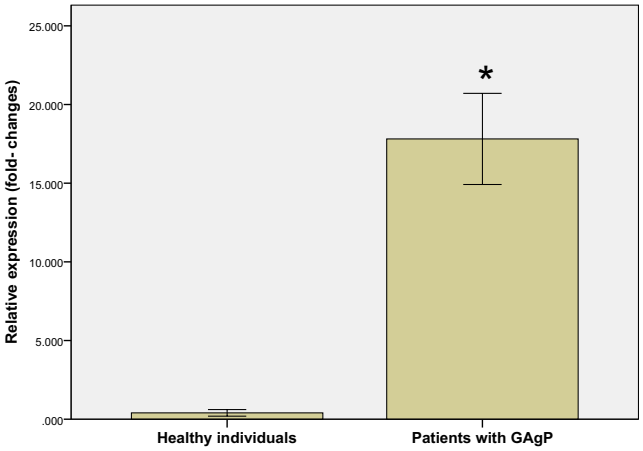

FIGURE 1 Levels of microRNA-146a (miR-146a) in gingival tissues from patients with GAgP. Subjects with GAgP had a higher level of miR-146a in their gingival tissues compared with subjects in the control group. Data are shown as mean ± standard error of the mean (SEM) of relative expression of miR-146a. *$P \leq .05$

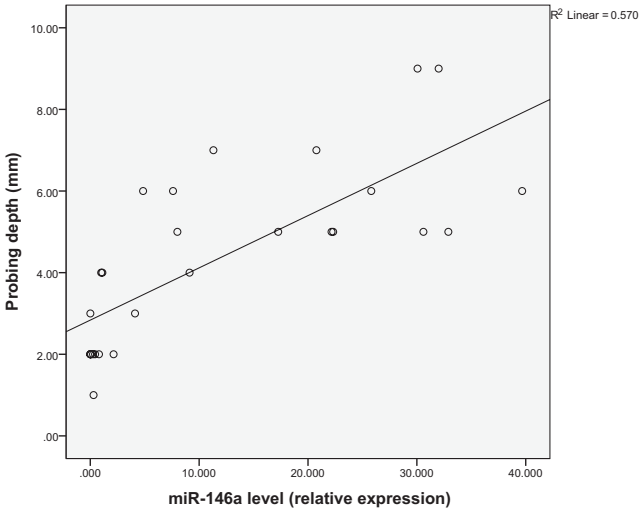

FIGURE 2 Association between the level of microRNA-146a (miR-146a) expressed and clinical parameters in patients with generalized aggressive periodontitis (GAgP). Spearman's correlation test showed a positive correlation between the miR-146a expression level and probing depth in patients. *$P \leq .05$

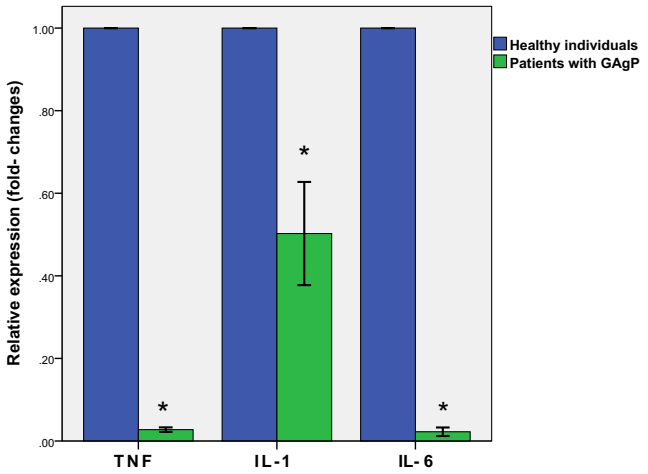

FIGURE 3 Pro-inflammatory cytokine levels in gingival tissues from patients with generalized aggressive periodontitis (GAgP). Quantification of cytokine levels in gingival tissues of patients showed a significant reduction in the levels of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). All data are shown as mean ± standard error of the mean (SEM) of the relative expression of cytokines. *$P \leq .05$

an established ethologic role of the inflammatory response in the pathogenesis of periodontitis, we observed a significant decrease of TNF-α, IL-1β, and IL-6 compared with healthy control subjects following Phase I periodontal therapy ($P \leq .05$) (Figure 3).

4 | DISCUSSION

miRNAs, which are regulators of gene expression post-transcription, play crucial roles in the physiological process.[13] Among them, miR-146a serves as a key negative regulator of the innate immune response.[20] Regarding the implication of miR-146a in human inflammatory disorders owing to its role in the regulation of inflammation, we sought to determine whether miR-146a expression has any potential link to the disease severity in GAgP.

In this study, we evaluated the miR-146a level in the gingival tissues of patients with aggressive periodontitis. Our results demonstrated a massively increased expression of miR-146a in the periodontal tissues obtained from patients with GAgP during surgical treatment.

Consistently, we also observed increased expression of miR-146a in patients with the chronic form of periodontitis.[1] Of course, given the difference in nature of these two diseases, comparison of the results is not logical. Subsequently, we evaluated the regulatory role of miR-146a in gene expression through assessing the expression of its targets. Among them, targets of proteins operating in the signaling of toll-like receptors and cytokine receptors (including TNF-α, IL-1β, and IL-6) were assessed.[6,20] According to our findings, overexpression of miR-146a in patients with GAgP was accompanied by a reduction in expression of these targets; we also found the same results in chronic periodontitis, although this disease is very different from aggressive periodontitis.[1] This difference may be a result of the regulatory role of miR-146a on production of pro-inflammatory cytokines,[13,20] but direct correlations between miR-146a expression and clinical parameters do not support a regulatory role of miR-146. In fact, this suggests that miR-146a may contribute to the pathogenesis of disease and/or serve as an indicator of disease severity in GAgP. Based on the current data, as stated by Takahashi et al[27], the immunological risk factors in generalized or localized aggressive periodontitis vary widely,[25,28-31] so by considering the expression of only a few cytokines we cannot conclude that in GAgP there is a reduction in expression of pro-inflammatory cytokines; in addition, the mRNA of some inflammatory cytokines, such as TNF-α, has a very short half life.[31]

It also has been shown that in response to inflammatory cytokines and bacterial components, such as LPS, miR-146a is upregulated in monocytes.[20] miR-146a, in turn, contributes to the downregulation of IL-1 receptor-associated kinase-1 and tumor necrosis factor receptor-associated factor 6, which are adaptor proteins operating downstream of toll-like receptors and cytokine receptors.[20] This leads to abolished activation of nuclear factor-kappa B, a key transcription factor implicated in transcription of many genes involved in inflammation, such as *TNFα*, *IL-1β*, *IL-6*, and *IL-8*, and chemokines, adhesion molecules, and prostaglandins.[6] We hypothesize that in aggressive periodontitis, bacterial components of plaque, particularly LPS, stimulate toll-like receptors, leading to elevation of the miR-146a level, which subsequently controls the level of pro-inflammatory cytokines through a negative-feedback loop.

Given the essential role of inflammation in the pathogenesis of periodontitis,[34] the unexpected finding of decreased expression of TNF-α, IL-1β, and IL-6, which are major pro-inflammatory cytokines, points to the contribution of other inflammatory mediators and/or a nonimmunologic-dependent mechanism in progression of the disease following Phase I periodontal therapy.

On other hand, it was shown that miR-146a participates in the regulation of many genes; for instance, downregulation of epidermal growth factor receptor and transforming growth factor beta;[33-35] so, it is also likely that miR-146a disrupts periodontal tissue repair and regeneration induced by both epidermal growth factor receptor and transforming growth factor beta.[33,34]

5 | CONCLUSION

Taken together, our results demonstrate that there is an association between miR-146a and GAgP and suggest that miR-146a may serve as an indicator of disease severity. However, more robust studies and studies with longitudinal design are required to clarify this association and the precise role of miR-146a in the development and/or progression of periodontitis.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

M. Sattari http://orcid.org/0000-0002-9152-981X

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