Analysis of Salivary MicroRNA-146a and IL-17 A in Chronic Periodontitis

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Abstract

Background: Periodontitis is a multifactorial disease that involves microorganism and host response causing periodontal tissue damages. MiRNAs is one of the epigenetic factors deregulation and many of them can contribute to the periodontitis pathogenesis. The current study aimed to investigate the level of miRNA-146a and IL-17A in saliva of chronic periodontitis patients and controls. This case-control study was performed on (40) chronic periodontitis patients and (40) healthy individuals as controls. Periodontal parameters used in the present study included PLI, GI, PPD and CAL. Saliva samples were taken from all subjects. ELISA was carried out to estimate the levels of IL-17, while miRNA-146a was determined by RTqPCR. The current results found a significant elevation in median salivary levels of IL-17 in patients group when compared with the control group (P˂ 0.05). Moreover, IL-17 showed a highly significant positive correlation with PLI, PPD & CAL. However, miRNA-146a revealed a significant down-regulation in the fold change of gene expression among the patients as compared to the controls (P˂ 0.05). Interestingly, significant strong inverse correlations were seen between miRNA-146a and both of PPD and CAL, as well as a weak inverse non-significant correlation between IL-17 and miRNA-146a. In Conclusion, these findings indicated that down-regulation of miRNA-146a with its negative correlation with periodontal parameters and IL-17 level in patients may aggravate the inflammatory immune response and contribute to pathogenesis of periodontitis.

Key words: MiRNA-146a, chronic periodontitis, Cytokines, IL-17.

Introduction

Periodontitis is a multifactorial oral inflammatory disorder characterized by progressive destruction of bone and ultimate tooth loss. It is initiated by microorganisms and further affected by environmental factors (1). Activation of the host immune response triggered by the pathogens and their metabolites stimulates the synthesis and release of cytokines and pro-inflammatory mediators resulting in tissue destructions (2). Cytokines are soluble proteins produced by cells that function as messengers to transmit signals to other cells. They mediate and control the inflammatory and immune responses and can regulate cellular differentiation & growth (3, 4). IL-17 is T cell derived proinflammatory cytokine and is a strong agent of inflammation in inflammatory conditions including periodontitis (5). It contributes to the pathophysiology of periodontitis, aggravating gingival inflammation and alveolar bone loss (6). IL-17 aggravates periodontal diseases through gingival fibroblast activation to secrete inflammatory cytokines. IL-17 has an action on alveolar bone cells, and it was documented that T-cells can directly contribute to bone metabolisms through T-cell derived cytokines including IL-17 (7).
Micro ribonucleic acid (miRNA) plays significant roles in diverse cellular biological processes like inflammatory proliferation, differentiation and response, and in carcinogenesis (8). They are short non-coding RNAs which act either by mRNA destabilizations or translational inhibition via sequence-specific binding sites within mRNA 3’-UTR of the gene. The target gene expressions are affected by MiRNAs through the fine-tuning and modulation of the protein expressions levels rather than the simple on or off gene-switching (9). The functions of oral epithelial and immune cells in periodontal diseases are affected by oral bacteria & inflammatory conditions and miRNA expression is dysregulated in such cells. While miRNAs are synthesized by immune and non-immune cells in the cells, they are also actively released by these cells into the extracellular environments such as extracellular fluid (10). miRNA has an important role in Porphyromonas gingivalis (P.g) virulence, especially in its contribution to modulate the host-cell immune response as it promotes bacterial survival & progressively decreases the protective action of the host. It is rather surprising that some of the miRNAs are also shown to be correlated with Porphyromonas gingivalis itself (11).

Two evolutionarily preserved members compose the miRNA-146 family, which are miRNA-146a & miRNA-146b; these members are situated on various chromosomes. The biologic activity of miR-146a & miR-146b seems not to be the same despite the huge structural similarities between them (12). When LPS is stimulated, the miR-146a is shown to be formed by a nuclear factor Kappa B dependent manner, resulting in innate immune response negative regulation through the down regulation of pro-inflammatory cytokine, chemokine & other inflammatory mediators (13). Therefore, our study aimed to investigate the level of miRNA-146a and IL-17A in the saliva of patients with chronic periodontitis and in controls.

Materials and Methods
In this study, (40) patients with chronic periodontitis whose ages ranged from (23-55) and a mean age of (37.67±1.57) years were enrolled, while the control group included (40) healthy subjects whose ages ranged from (25-49) years and a mean age of (34.70±1.25) years. The periodontal parameters estimated in this study included PLI, GI, PPD & CAL. Detailed information were obtained from each subject concerning the nature of the study and the procedures included, and their informed consent was acquired on a form authenticated by the ethical committee at the College of Dentistry / University of Baghdad.

Saliva sample collection
All subjects included in this study were informed not to eat or drink (except water) at least an hour before saliva collection. The subjects were instructed to sit relaxed and blood-containing samples were neglected. Saliva specimens’ were obtained between 9-12 am. After rinsing their mouths many times with sterilized water and waiting for (1-2) minutes for water clearance, about (5) ml of the unstimulated saliva were placed in polyethylene tubes. The collected saliva then were spun in the centrifuge at (3000) RPM for (10) minutes and the resulting supernatant was kept at (-80°C) in Eppendorf tubes for further processing.

Determination of IL-17
The level of IL-17A was determined by the commercially-available ELISA kit and done according to the guidelines present in the attached leaflet (Shanghai/China).
Saliva RNA extraction
The purification of total RNA, including miRNA was performed by the use of the TRIzol® Reagent in accordance with the guidelines of the manufacturer (Qubit™ microRNA HS assay kit, ThermoFisher, USA). Then, RNA concentration and purity was determined with Qubit® Fluorometer.

Reverse Transcriptase
The ProtoScript® kit was utilized to synthesize the complementary DNA (cDNA) by using purified total RNA containing miRNA as a template. In this step, (5) µl from each extracted total RNA sample was added into new PCR tube. Protoscript reaction mix that contains dNTPs, buffer and other essential components were added as 10ul for each sample. MuLV enzyme was then added into the reaction as 2ul per sample. Two µl from specific primer was added, and the volume was completed up to 20ul by adding 1ul. This mixture was incubated for (1) hour at (42) ºC by using thermocycler and this was followed by (80) ºC for inactivation of enzyme. The cDNA product quantification was also done through Qubit and stored until performing the second step (Relative quantitative PCR).

Quantitative PCR (qPCR)
According to protocol of the manufacturer, cDNA prepared in the previous step, served as a template for miRNA-146a quantification, by RT-PCR System (Bioer/ Japan) together with (Luna Universal qPCR Kit; NEB/UK); and hsa-miRNA-146a (F- 5'-GGGTGAGAACTGAATTCCA-3'; R- 5'-CAGTGCGTGTCGTGGAGT-3') and U6 snRNA (F- 5'-GGGTGAGAACTGAATTCCA-3'; R- 5'-CAGTGCGTGTCGTGGAGT-3') primers.

Statistical analysis: The T-test and Mann-Whitney tests were utilized to calculate the statistical significance of difference between groups, while the Spearman and Pearson correlation coefficient tests were used to calculate correlations among different parameters. The P-values of P<0.01 and P<0.05 have been regarded as significant.

Results
The demographic characteristics of the patient and control groups are illustrated in table (1). The patient's mean age was (37.67±1.57) years, while the control's mean age was (34.70±1.25) years. There was a predominance of females (67.5%) in comparison with males (32.5%) among chronic periodontitis patients. No significant difference (P>0.05) was found between the two study groups (patients and controls) in regard to age and gender.

Table (2) shows the difference in periodontal parameters between patients and control subjects.
Table (1): Demographic characteristic of patients & controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>The patient group</th>
<th>The control group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Range</td>
<td>23-55</td>
<td>25-49</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>37.67</td>
<td>34.70</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.57</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>9.97</td>
<td>7.96</td>
</tr>
<tr>
<td>Gender</td>
<td>Male No.</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>% (32.5%)</td>
<td>(40%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female No.</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>% (67.5%)</td>
<td>(60%)</td>
<td></td>
</tr>
</tbody>
</table>

Table (2): Clinical periodontal parameters of patients & controls

<table>
<thead>
<tr>
<th></th>
<th>Patients group</th>
<th>Controls group</th>
<th>T-test(p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLI</td>
<td>1.24±0.05</td>
<td>0.58±0.04</td>
<td>0.000&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>GI</td>
<td>1.38±0.06</td>
<td>0.24±0.02</td>
<td>0.000&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>PPD (mm)</td>
<td>4.74±0.08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAL</td>
<td>4.91±0.20</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The current results found significant elevation in salivary IL-17A concentrations among chronic periodontitis subjects, with median (44.32 ng/L) when compared to the healthy controls (36.25 ng/L), (P< 0.05) as shown in table (3).

Table (3): Salivary median levels of IL-17A (ng/L) in patient & controls

<table>
<thead>
<tr>
<th>Salivary IL-17A</th>
<th>Patients group</th>
<th>Controls group</th>
<th>Mann-Whitney p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>22.45</td>
<td>11.41</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>215.71</td>
<td>105.68</td>
<td></td>
</tr>
<tr>
<td>Mean Rank</td>
<td>46.31</td>
<td>34.69</td>
<td>0.0257</td>
</tr>
<tr>
<td>Median</td>
<td>44.32</td>
<td>36.25</td>
<td></td>
</tr>
</tbody>
</table>

Spearman’s correlation between salivary IL-17A levels & periodontal parameters in patients shows that there is highly significant positive association between salivary IL-17A levels and PLI, PPD & CAL levels (r=0.436, p=0.004; r=0.534, p=0.000 and r=0.595, p=0.000) consecutively as observed in table (4).
As shown in table (5), the mean ΔCt value of miRNA-146a in chronic periodontitis cases (2.70± 0.82) was higher than its value in the healthy control group (0.92± 0.35), which means it was down regulated with 0.74 fold change, with a statistically significant variation (p<0.05).

This study found significant strong inverse correlations between salivary miRNA-146a levels and PPD and CAL levels (r= -0.417, p=0.024, r= -0.591, p= 0.011) as demonstrated in table (6). Furthermore, figure (1) showed the linear regression correlation between salivary IL-17 with MiRNA 146a which revealed a weak inverse non significant correlations (r= -0.276, P=0.319) in chronic periodontitis patients.
Discussion

Chronic periodontitis is a complex chronic inflammatory microbial disorder induced by a group of pathogenic microorganisms, which leads to inflammation and the destruction of periodontal tissues (14). Up to our knowledge, it’s the first study in Iraq to assess the role of miRNA-146a & its target IL-17 in the pathogenicity of chronic periodontitis. IL-17 is a proinflammatory cytokine which plays an essential role in the chronic periodontitis pathogenesis (15). The current study showed that the median saliva level of IL-17 was higher in chronic periodontitis patients than the healthy control group. The higher level of this cytokine in patients may be considered as expected since the IL-17 cytokine is postulated as a proinflammatory mediator involved in inflammatory response (16). Furthermore, Batool and colleagues reported that the level of salivary IL-17 raised significantly in calculus-related chronic periodontitis patients compared to the healthy controls & this level raised as the chronic periodontitis progressed. So they concluded that salivary IL-6 levels can help in the subcategorization of chronic periodontitis (17). The production of high IL-17 amounts can have an effect on pathogenic role of periodontitis. IL-17 can cause elevated inflammation via further recruitment of neutrophils, through raising the proinflammatory cytokine productions (18).

Other studies performed by Avani et al on Indian population disagreed with these findings when they revealed that IL-17 concentrations among chronic periodontitis patients was not elevated than that in control (19). Also Pradeep et al. revealed that IL-17 levels could not be estimated in detectable levels in GCF of healthy individuals, patients with gingivitis and mild chronic periodontitis. There was a significant positive correlations between salivary IL-17 levels and the means of PLI, PPD & CAL, and these findings were consistent with (20) who detected a positive relationship between IL-17 levels and the patient's periodontal condition. Similarly Lester et al. (2007) demonstrated a positive correlation between the tissue concentration of IL-17 cytokine and CAL. While the present finding was contradicted with other finding that showed no significant correlation between serum IL-17 conc. and mean PPD & CAL conc. in individuals diagnosed with chronic periodontitis (21).
MiRNA-146a acts as a main negative innate immune response regulator. As regard the involvement of miR-146a in inflammatory disease due to its role in inflammation regulation, so this study establishes to find whether miRNA-146a expression has a correlation with disease severity in chronic periodontitis. The current study revealed a significant variation in the microRNA-146a gene expression in saliva specimens of chronic periodontitis patients in comparison with the control with 0.74 fold of expression. The current finding agreed with another result that showed that miRNA-146a was down-regulated among periodontitis patients in comparison with the healthy controls (22). Boldin et al. revealed that an in vivo deficiency of miRNA-146a may induce macrophage hyper-activation, increase systemic response to the endotoxin (LPS) & predispose in an autoimmune phenotype development later in life (23). Other study postulated that the miRNA-146a down regulation or its impaired function can be correlated with a disease, e.g. aggressive periodontitis, where sustained or exaggerated inflammation participate in the pathogenesis process (24). Other type of miRNA which is miRNA-196a, its gene expression was studied among chronic periodontitis individuals and showed significant inverse association with disease (25).

On the contrary, results reported by Gao and Hao found that the expression of miRNA-146a in salivary supernatant of chronic periodontitis patients is significant higher than that normal subjects, and it is significantly decreased after periodontal treatment, suggesting that salivary miRNA-146a can be used as potential target to prevent and treat periodontal disease (26). Whether it is up-regulated or down-regulated in periodontal diseases, miRNA-146a is shown to play functional main roles in this disease (27, 28). Nonetheless the discrepancy of results among various studies might be due to the differences in the ethnicity and experimental conditions.

However, Jiang et al. demonstrated that levels of miRNA-146a in B cell were increased by the stimulation of P. g LPS, and the level of expression of IL-1β, IL-6, IL-10 & IRAK1 were decreased in the existence of miRNA-146a mimic, while they were increased after miRNA-146a inhibitor addition. Moreover, overexpression of IRAK1 inverted the inhibitory effect of miR-146a on IL-1β, IL-6 & IL-10. So they concluded that miRNA-146a causes inhibition of inflammatory cytokines production in B-cells by direct targeting of IRAK1, assuming a regulatory action of miR-146a during the periodontal inflammation of B-cell-mediated (29).

Next, this study evaluated the relationships between the relative expressions of the miRNA-146a and clinical parameters by Pearson’s correlation test. Significant strong negative correlation between salivary miRNA-146a levels and each of PPD & CAL was shown in our study. In fact, this inverse correlation between the expression of miRNA-146a and the clinical parameter support the regulatory roles of miRNA-146a. In contrast, Motedayyen et al. reported positive correlation between the levels of miRNA-146a and the clinical parameters and assumes a pathophysiologic roles of miR-146a in chronic periodontitis (30).

Subsequently, the present study evaluates a miRNA-146a regulatory role in gene expression by evaluation of the level of its target (IL-17A). Based on data in this study the linear regression between IL-17 and miRNA-146a which revealed non-significant inverse correlation between them in chronic periodontitis patients. This result was consistent with other result reported by (22) who pointed out an inverse correlation between miRNA-146a
and IL17 in periodontitis subjects. Thus the study concluded that miRNA-146a may improve periodontitis by down-regulating IL-17 expression.

This inverse correlation may be due to the low level and the weakened ability of miRNA-146a, which also resulted in the elevation of IL-17, may be involved in the progression of periodontitis. Likewise Motedayyen et al. stated that there is inverse correlation between miRNA-146a and pro-inflammatory cytokines, and observed that elevated miRNA-146a level accompanied a significant decrease in TNF-α & IL-6 levels\(^{(30)}\). In conclusion these findings indicated that down-regulation of miRNA-146a with its negative correlation with periodontal parameters and IL-17 level in patients may aggravate the inflammatory immune response and contribute to pathogenesis of periodontitis.

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**Conflict of interest:** None.

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