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Dysregulation of human miRNAs and increased prevalence of HHV miRNAs in obese periodontitis subjects

Afsar R. Naqvi[1,*], **Maria F. Brambila**[2], **Gloria Martínez**[2], **Gabriela Chapa**[2], and **Salvador Nares**[1,*]

[1]Department of Periodontics, University of Illinois at Chicago, Chicago, IL, USA.

[2]Posgrado de Periodoncia, Facultad de Odontologia, Universidad Autonoma de Nuevo León, Monterrey, Mexico.

Abstract

Aim: To evaluate human and herpesvirus-encoded microRNA (miRNA) expression in healthy and diseased gingiva of obese and non-obese subjects and compare the impact of localized and systemic inflammation on human miRNA profiles.

Material and methods: Healthy and inflamed gingival biopsies were collected from obese and non-obese subjects. Human and herpesvirus miRNA expression was quantified using quantitative PCR. Predicted targets of dysregulated miRNAs were identified using bioinformatics analysis, validated by dual luciferase assays and their expression assessed in healthy and diseased tissues.

Results: Our results show differential expression of miRNAs in both diseased groups compared to healthy counterparts. MMP-16 is identified as a novel target of miRNAs altered in disease. Expression analysis of genes predicted as target of differentially expressed miRNAs show significant changes in disease compared with healthy tissues. Finally, quantitation of four herpesvirus derived viral miRNAs show that expression and prevalence of herpesvirus miRNAs in diseased gingiva of obese subjects.

Conclusion: Our findings show that miRNA (both cellular and virus) expression are differentially responsive to local and systemic inflammation. Some of these miRNAs can modulate key cellular genes with direct consequences on inflammatory pathways suggesting their impact on oral tissue transcriptome and functions.

Keywords

Obesity; periodontitis; microRNA; viral microRNA; membrane metalloproteases; immunity

[*]**Correspondence to:** Dr. Salvador Nares, University of Illinois at Chicago, College of Dentistry, Department of Periodontics, Dent MC 859, 801 South Paulina, Chicago, IL 60612. snares@uic.edu.

Author Contributions

A.R. Naqvi contributed to conception, design, data acquisition, and analysis, drafted the manuscript; M. Navarrete and G.M. Sandoval contributed to sample collection, conception, design of study; S. Nares contributed to conception, sample collection, design, drafted the manuscript; All authors gave final approval and agree to be accountable for all aspects of the work

Conflict of Interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article

Introduction

Periodontal diseases are a group of infectious, inflammatory disorders affecting the supporting tissues of teeth. The primary etiological agents are microbial in nature, predominantly bacterial, which form a plaque biofilm. Here an exuberant host response results in destruction of periodontal tissues and can culminate in tooth loss. Genetic, behavioral, and environmental risk factors including heredity, smoking, stress, and obesity have been associated with onset, progression, and severity of disease (Loos et al., 2015; Nociti et al., 2015; Newton & Asimakopoulou, 2015). However, the underlying mechanisms associated with periodontal pathology are not completely understood.

Obesity is a chronic inflammatory condition with worldwide prevalence, and numerous studies have described the impact of obesity in the pathophysiology of periodontal disease. Studies have reported that obesity is associated with greater prevalence and severity of disease (Gorman et al., 2012; Gorman et al., 2012; Linden et al., 2007; Jimenez et al., 2012) while, mechanistic studies have revealed that macrophages exposed to a hyperlipidemic environment display altered functional capacity including reduced infiltration, activation and phagocytic capacity (Huang et al., 2016), defective oxidative burst (Lee et al., 1999; Mancuso et al., 2002) and decreased macrophage cytokine production (Chu et al., 1999; Doxey et al., 1998).

Our understanding of the cellular and molecular mechanisms associated with periodontal disease has significantly improved over the last several decades. In particular, recent studies highlight the important role of non-coding RNA, particularly microRNA (miRNA) on modulation of gene expression and its functional impact on immunity (Lee et al., 2016; Jia et al., 2014; Naqvi et al., 2016; Luan et al., 2018), inflammation (Lee et al., 2016; Alexander & O'Connell, 2015; Naqvi et al., 2015; Naqvi et al., 2016), and osteoclastogenesis (Ji et al., 2016; Tang et al., 2014; Fordham et al., 2016), key aspects of periodontal pathology. We and others have reported miRNA profiles in gingival biopsies of healthy and diseased periodontal tissues (Motedayyen et al., 2016; Kalea et al., 2015; Ogata et al., 2014; Stoecklin-Wasmer et al., 2012; Perri et al., 2012; Lee et al., 2011) and response of cells to lipopolysaccharide (LPS) from periodontal pathogens (Huck et al., 2017; Chen et al., 2016; Fordham et al., 2015; Naqvi et al., 2014). Together, these *in-vivo* and *in-vitro* studies have confirmed divergent miRNA profiles under inflamed conditions and that altered expression of miRNA can have functional consequences by direct regulation of genes involved in key pathways.

In addition to dysregulation of miRNA profiles, we and others have reported that systemic conditions such as obesity can further dysregulate miRNA profiles in human gingiva (Kalea et al., 2015; Perri et al., 2012). These studies highlight the impact of obesity on gene regulation in periodontal tissues. However, as miRNA can modulate both immune and inflammatory gene targets, characterizing the effects of chronic periodontal inflammation in the context of obesity is necessary to fully comprehend how systemic conditions contribute to periodontal pathology. Here we examined the miRNA profiles and four previously reported herpesvirus-encoded miRNAs in healthy and diseased gingiva of obese and non-

obese subjects. We validated two novel target genes of differentially expressed miRNAs and note that genes and miRNAs exhibited antagonistic expression.

Materials and Methods

Study population and sample collection

This study was approved by the Ethics Committee at the University Autónoma de Nuevo León Facultad de Odontología and The University of Illinois at Chicago, College of Dentistry. Subjects presenting to the Postgraduate Periodontics Clinic at the Dental School of the Universidad Autonóma de Nuevo León were recruited for this study. Obese and non-obese subjects with healthy and diseased periodontium (total four study groups) were recruited for this based on BMI (non-obese BMI < 30 kg/m^2 and obese BMI > 30 kg/m^2) and the presence or absence of chronic periodontitis as previously described (Perri et al., 2012). Subjects with chronic periodontal disease displayed probing depth ≥ 6mm with bleeding on probing and radiographic evidence of bone loss. Health periodontal patients displayed probing depths ≤ 3mm, with no bleeding on probing and no radiographic evidence of bone loss. Inclusion criteria included male and female patients ages 18 to 65 years and in good systemic health. Exclusion criteria included chronic disease (diabetes, hepatitis, renal failure, clotting disorders, HIV, etc), antibiotic therapy for any medical or dental condition within a month before screening, and subjects taking medications known to affect periodontal status (e.g. phenytoin, calcium channel blockers, cyclosporine). For periodontally healthy (obese/non-obese) subjects (N=14/group), a single gingival biopsy sample (including gingival epithelium, col area, and underlying connective tissue) was collected at the time of crown-lengthening procedures. The biopsy sample was harvested using intrasulcular and inverse bevel incisions approximately 2 mm from the free gingival margin at the crest of the interproximal papillae extending horizontally capturing the interproximal col area and immediately placed in RNAlater (Qiagen, Gaithersburg, MD, USA) and stored at −80°C. For subjects (obese/non-obese) with chronic periodontitis (N=14/group), a similar gingival biopsy sample was collected at the time of periodontal flap surgery.

Tissue lysis and RNA preparation

Samples were thawed on ice and tissue lysis was performed as described previously (Perri et al. 2012). Total RNA (including miRNAs) was isolated using the miRNeasy kit (Qiagen) according to manufacturer's instructions.

Reverse transcription and real-time PCR

One microgram of total RNA from each sample was reverse transcribed to cDNA (20μl reaction) using miScript II RT Kit (Qiagen) as per manufacturer's instructions. MiRNA PCR array data was analyzed using Qiagen GeneGlobe Data Analysis Center (https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/). Expression levels of miRNAs across the different groups were normalized with respect to at least three most consistently expressed endogenous controls in all samples. Next, the fold change was calculated with respect to the healthy control. miRNAs identified as significantly altered by software analysis were included as differentially expressed miRNAs.

miR-24 and miR-27a level was validated in a separate cohort of N=10/group using similar conditions. Expression of mRNAs (N=10/group) was analyzed as described above using gene specific primers. Four herpesviral miRNAs (miR-H1, miR-US4, miR-K12-3 and miR-UL70-3p) were quantified in the same cohort. RNU6 and GAPDH served as miRNA and mRNA normalization controls.

miRNA targeted pathway and gene prediction analysis

MiRNA targeted pathways were predicted using DIANA-miRPath (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index). We selected DIANA-microT-CDS algorithm that included predicted miRNA targets. To identify the potential miR-24 and miR-27a binding sites, miRwalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) prediction tool was used. We selected 10 different algorithms tools to identify potential targets.

Cell culture and transient miRNA transfections

MiR-24, miR-26a, miR-27a, miR-30b or control mimics were purchased (Qiagen) and transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in HEK293 and human oral keratinocytes as previously described (Naqvi et al., 2018).

Luciferase reporter constructs and dual luciferase reporter assays

Cloning of the predicted gene 3′UTRs and dual luciferase assays was performed as previously described (Naqvi et al., 2015). Target gene region was cloned in psiCHECK2 vector using specific set of primers (MMP-16 Forward: GCACTCGAGTGACCTTTCAAACCCAGAGG and MMP-16 Reverse: ATGCGGCCGCTTCAGTTTGTGCCAGTTTGC and MAPK1 Forward: GCACTCGAGCGGAAAACAGACCCACATCT and MAPK1 Reverse: ATGCGGCCGCAGGAACAGCTCACAGCCCTA).

Statistical Analysis

Data were analyzed on GraphPad Prism (GraphPad Software, La Jolla, USA). Results are represented as standard deviation or ±SEM from three independent replicates and experiments were conducted at least thrice. P-values were calculated using Students t-test and $p < 0.05$ were considered significant.

Results

Altered miRNA profiles in health and diseased gingiva

Because we were interested in comparing the effects of systemic inflammation on periodontal health, we collected gingival biopsies from periodontally healthy and diseased obese human subjects. For each group, six subjects were recruited into the study. Age and gender distribution was assessed by Mann-Whitney U test and McNemar $\chi 2$ test in healthy and disease groups, respectively. No difference were noted in age (p=0.42) or sex (p=0.55) distribution between the obese and non-obese groups (see Supplemental Material Table S1).

Comparison of obese subjects with healthy and diseased gingival tissues identified thirty-nine upregulated and two downregulated miRNAs ($p<0.05$). The fold change for upregulated miRNAs was set at ≥1.6 and for downregulated was ≤0.52. MiR-21-5p, let-7f-5p, miR-29b/c, miR-24-3p, miR-27a-3p were among the upregulated miRNAs, while miR-423-5p and miR-196b-5p showed reduced expression levels. The list of differentially expressed miRNAs is provided in Table 1.

Comparison of miRNA expression in healthy and diseased gingival tissue in non-obese subjects showed one upregulated (miR-150-5p) and twenty downregulated (e.g., miR-26b-3p, miR-27b-3p, miR-185-5p) miRNAs with similar cut-off for fold-change and p-value (Table 2). We noticed 16 dysregulated miRNAs that were common to obese and non-obese datasets. However, all displayed antagonistic fold change expression compared to their corresponding controls.

We analyzed the differences in miRNA expression between obese and non-obese, periodontitis and healthy subjects. Several miRNAs exhibited significant differences in both analysis (Supplemental Material Table S2 and S3). Compared to non-obese periodontitis subjects, sixteen upregulated miRNAs were identified in obese periodontitis subjects (Supplemental Material Table S2). Similarly, comparison of miRNA profiles in obese and non-obese healthy subjects showed one upregulated and twenty-three downregulated miRNAs (Supplemental Material Table S3).

The expression of miR-24 and miR-27a were further validated in a separate cohort of periodontally healthy and diseased obese and non-obese subjects (N=10/group). In obese subjects, higher expression of miR-24 (~4 fold; $p<0.0001$) and miR-27a (~10 fold; $p<0.0001$) was observed in inflamed gingiva compared to healthy gingiva (Figure 1A and B). Conversely, downregulation of both miR-24 (~1.6 fold; $p<0.0001$) and miR-27a (~2.5 fold; $p<0.0001$) expression was observed in gingiva from non-obese subjects with periodontal inflammation compared to healthy gingiva (Figure 1A and B). These results corroborate with the PCR array data and further indicate that miRNA profiles in obese and non-obese healthy and inflamed gingiva are markedly distinct.

Global pathway analysis of altered miRNAs

To evaluate the biological impact of altered miRNA levels on the pathogenesis of disease, differentially expressed miRNAs from each dataset were subjected to pathway analysis. Table 3 shows a list of selected common and unique relevant biological pathways with significant FDR corrected p-values (<0.01) for the differentially expressed miRNAs from obese and non-obese datasets. Several pathways related to cytokine and transcription factor (TNF, TGF-β, p53) signaling, PI3K-Akt signaling, apoptosis, adhesion and junction molecules, ECM-receptor interaction, various cancer associated pathways were commonly identified in both datasets. This correlates with the common set of miRNAs dysregulated in both non-obese and obese periodontal tissues (Table 3).

We also noted pathways unique to miRNAs identified in obese and non-obese periodontitis subjects (Table 3 and Supplemental Material Table S4). Among the 77 different pathways identified in obese periodontitis dataset, eleven pathways were unique. Intriguingly, many of

these pathways were related to lipid biosynthesis or metabolism. These included biosynthesis of steroid, unsaturated fatty acids, and fatty acid degradation pathways. Similarly, the non-obese dataset shows 12 unique pathways which were mostly related to bacterial (amoebiasis, *H. pylori*) and viral (EBV, HCV infection) infection, pathogen clearance (Fc gamma R-mediated phagocytosis, regulation of actin cytoskeleton) or signaling (MAPK and AMPK signaling).

Expression profiles of predicted targets of dysregulated miRNAs

We focused on genes with functions related to inflammation and wound repair related pathways, key elements of periodontal pathogenesis. With these criteria, matrix metalloproteinase 16 (MMP-16) and mitogen-activated protein kinase 1 (MAPK1; also known as ERK2) were selected as target genes. The 3′UTR of MMP-16 displays predicted sites for miR-24, miR-26a and miR-27a, while MAPK1 harbors complementary sites for miR-27a and miR-30b. The expression of these miRNAs was increased in gingival biopsies derived from obese, periodontally diseased subjects while expression in non-obese periodontitis subjects was downregulated (see Table 1 and 2). Expression of both MAPK1 and MMP-16, predicted targets of miR-24 and miR-27 (induced in obese periodontitis), were downregulated in obese periodontitis subjects compared to healthy counterparts (Figure 1C, D and Table 1). In non-obese subjects, increased levels of MAPK1 and MMP-16 expression was noted in diseased gingiva that corroborated with reduced levels of miR-27a and miR-24 in the same tissues (Figure 1 C, D and Table 2).

miR-24 and miR-27a directly target MMP-16

To validate the antagonistic expression of miRNAs and MAPK1 and MMP-16, we cloned the 3′UTR of MMP-16 and MAPK1 encompassing the miRNA binding sites. Figure 2 (A, D) shows sequence alignment of miRNA and the target genes. MMP-16 harbors binding sites for three differentially expressed miRNAs viz., miR-24, miR-26a and miR-27a (Figure 2A), while MAPK1 harbors a predicted binding site for miR-27a and miR-30b (Figure 2D). Because the MAPK1 3′UTR was comparatively long (~4.6 kb), we only cloned ~1950 nts which encompasses miR-27a and miR-30b binding sites (Figure 2D). Using dual luciferase assays, we screened the functional miRNA and target gene interaction in HEK293 cells. Our results show that miR-24 downregulates reporter (renilla luciferase) gene activity compared to control mimic or empty vector transfected with miR-24 (Figure 2B). This miRNA-target interaction was confirmed in a second cell line *viz.*, HeLa (Figure 2B). We also screened miR-26a and miR-27a interaction with MMP-16 in HEK293 cells and observed that miR-27a, but not miR-26a downregulated renilla activity compared to control mimic (Figure 2C).

MAPK1 and its predicted interacting miRNAs, miR-27a and miR-30b were also screened by dual luciferase assays (Figure 2D). Compared with control mimic, cells transfected with miR-27a but not miR-30b, showed significantly reduced renilla activity confirming MAPK1 as a novel target of miR-27a (Figure 2E). Additionally, HGEC were transfected with miR-24, miR-26a, miR-27a or control mimic and expression of MMP-16 and MAPK1 transcripts was evaluated. Compared to control, significantly reduced expression of MMP-16 mRNA was observed in miR-24 but not miR-27a or miR-26a transfected cells, while

expression of MAPK1 was significant downregulated by miR-24 and miR-26a (Figure 2F and G).

Herpesvirus miRNAs detected in diseased periodontal tissues

To test whether local inflammation may influence v-miR expression, we examined expression of four previously identified v-miRs. Our results show that miR-H1 was detected in all obese periodontitis biopsies, while this miRNA was not detected in any of the tissue samples derived from obese, but periodontally healthy individuals (Figure 3A). For miR-US4, diseased tissue biopsies derived from obese patients showed higher prevalence and expression (~9 fold high) compared to respective obese, periodontally healthy subjects (Figure 3B and D). Similarly, miR-K12–3 was more prevalent and showed higher expression (~3.3 fold) in periodontally diseased, compared to healthy tissue samples (Figure 3C and E) from obese subjects. Expression of miR-UL70–3p was below detection limit (Ct values >36, data not shown).

Discussion

In this study, we show that miRNA profiles display convergent and divergent expression pattern in gingival biopsy samples derived from obese and non-obese periodontitis subjects. This is consistent with previous reports on independent and geographically distinct cohorts (Kalea et al., 2015; Ogata et al., 2014; Perri, et al., 2012). Besides various internal and external stimuli, miRNA is also responsive to periodontal pathogen-derived immunogenic components that trigger transcriptome changes in miRNA expression. We noted miRNAs that were common to previous studies. Three miRNAs (miR-15a, miR-30 family members and miR-142-3p) upregulated in our obese periodontitis data were also reported by Perri et al., (2012). Similarly, miR-150 (upregulated), miR-26a-5p and let-7 family members (downregulated) identified in our non-obese periodontitis data were common to Ogata et al., (2014). However, no overlap in altered miRNAs was observed between our dataset and Kalea et al., (2015). Multiple factors including ethnicity, extent of periodontal disease severity, and miRNA detection platforms can contribute to variations across different cohorts. Moreover, the increased infiltration of inflammatory cells and loss of structural cells (e.g., epithelial, fibroblasts, etc.) can directly contribute to the observed differences. We also acknowledge that the small sample size (N=6) used for PCR array analysis could be an additional factor contributing to the miRNA expression variability. However, validation of miR-24 and miR-27a levels in a second, independent cohort of specimens (N=10) revealed similar expression pattern as observed in the PCR array. We also validated expression of miR-26a in another cohort and observed similar expression pattern (Naqvi et al., unpublished results). Although not tested in this study, subjects with severe periodontal disease (≥ 6 mm probing depths) may exhibit different miRNA profiles compare to those with mild or moderate forms of periodontitis (4–5 mm probing depths).

Increased levels of miR-24 and miR-27a expression were observed in obese periodontitis subjects, while in non-obese subjects, the levels of miR-24 and miR-27b were downregulated compared to respective controls. Based on our dual luciferase and qPCR data, we show that MMP-16 is a novel target of miR-24 and miR-27a. In obese periodontitis

subjects, we noticed reduced MMP-16 levels while the non-obese periodontitis subjects showed high levels of MMP-16. Thus, miR-24 and miR-27a exhibit antagonistic expression to MMP-16 in gingival tissues. MMPs are a family of calcium-dependent zinc containing endopeptidases. They are involved in orchestrating many events related to the inflammatory response, migration of immune cells by processing the extracellular matrix, stimulation and immune-cell recruitment by processing chemokines, resolving the inflammatory process using the complement system, and enhancing phagocytosis (Page-McCaw et al., 2017; Nagase et al., 2006). The substrate for MMP-16 identified as a novel miRNAs target in this study is MMP-2, known to accelerate cell migration. MMP-2 works closely with MMP-9 to increase keratinocyte migration during tissue remodeling (Caley et al., 2015). Targeting of MMP-16 by miRNAs (miR-24 and miR-27a) in gingival samples derived from periodontitis subjects with obesity suggests that dysregulation of miRNAs can contribute to disease pathogenesis by altering key genes involved in tissue integrity and turnover.

Studies from various groups including ours have demonstrated that miR-24, miR-30b and miR-142-3p function as anti-inflammatory miRNAs (Naqvi et al., 2015; Sun et al., 2011; Fordham et al., 2015). In myeloid inflammatory cells, overexpression of these miRNAs inhibit innate and adaptive immune responses. In our comparative miRNA profiling, we noted increased levels of miR-24, −30b and −142-3p levels in obese periodontitis subjects suggesting that these miRNAs may impair the immune response and thus facilitate survival or spread of periodontal bacteria and possibly viruses. MMP expression is induced by inflammatory mediators like tumor necrosis factor (TNF-α), interleukin (IL)-1β, both of which play central role in the pathogenesis of periodontitis (Graves & Cochran 2003; Zhang et al., 2013). Hence, miR-24-mediated silencing of MMP-16 and suppression of inflammatory cytokines may act as a two-prong mechanism that can contribute to the periodontal pathogenesis.

Independent of the innate host response, periodontal pathogens target and disrupt various host cellular pathways including tissue homeostasis and repair that are of paramount importance to the pathogenesis of periodontal disease (Darveau, 2010; Pyrc et al., 2013; Quinchia-Rios et al., 2008). Extracellular signal-related kinases (Erks) are one of the three main families of MAP kinases. The classic Erk (Erk1/2) pathway is primarily activated upon growth factor or cytokine binding to the EGF receptor. The cascade of protein phosphorylation leads to activation of transcription factors (including Sp1, E2F, Elk-1, AP-1) that induce expression of genes involved in cell differentiation, proliferation and survival (Murphy & Blenis, 2006). For instance, *Porphyromonas gingivalis* (Pg) derived virulence factor peptidylarginine deiminase (PPAD) can impair biological activity of Epidermal Growth Factor (EGF) by citrullination of C-terminal arginine (Pyrc et al., 2013). This disrupts the activation of ERK pathway consequently leading to reduced epidermal cell proliferation. Similarly, Pg LPS is known to inhibit EGF-mediated signaling by reducing the phosphorylation of ERK2 (MAPK1) and other components of the MAPK signaling cascade including ERK1, p38 MAPK, and cyclic-AMP response element binding (CREB) (Quinchia-Rios et al., 2008). We identify and demonstrate that miR-24 and miR-27a and miR-30 target MAPK1 and exhibit antagonistic, yet differential expression in diseased obese and non-obese gingival tissues. MicroRNA-mediated post-transcriptional suppression of

MAPK1 expression in obese diseased gingiva can impair tissue repair mechanisms that may delay periodontal healing.

The presence of herpesviruses is increasingly acknowledged to exacerbate pathogenesis of oral inflammatory diseases including periodontitis (Parra & Slots 1996; Sabeti et al., 2003; Slots et al., 2003; Naqvi et al., 2018). However, the presence of the viral genome and its contribution to disease manifestation remains unanswered. To understand the biological relevance of these findings in periodontitis, we examined the expression of four previously identified viral miRNAs reported in our previous studies (Zhong et al., 2017; Naqvi et al., 2018). In biopsy samples derived from obese periodontally healthy and diseased subjects, quantification of v-miRs revealed higher levels of herpesviral miRNAs in diseased tissues compared with their healthy counterparts. These findings corroborates with our previous results in non-obese healthy samples and a closer analysis showed no significant differences between the two cohorts. This suggests that at least changes in the levels of these four v-miRs were altered in periodontitis but not impacted by systemic inflammation. Increase in the viral miRNAs levels indicate higher viral load in the inflamed oral tissues. Additionally, similar to cellular miRNAs, viral miRNAs can also post-transcriptionally regulate numerous host genes. We recently showed that viral miRNAs can alter expression of both host mRNAs and miRNAs in v-miR expressing oral keratinocytes and myeloid cells (macrophages) (Naqvi et al., 2018a-c). These widespread vmiR-mediated transcriptome changes can affect numerous cellular functions including immune responses, phagocytosis, cell migration, etc. Impaired immune response observed in v-miR transfected macrophages specifically demonstrate their capability to modulate immunity and bacterial clearance, both of which can significantly contribute to periodontal pathogenesis. These findings strongly suggest that in addition to alteration in cellular miRNA profiles in diseased gingiva, increased inflammation also perturb viral miRNAs profiles, which can modulate host cell functions as well as viral life cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Relevance

Scientific rationale for the study:

We sought to compare the expression profiles of human and herpesvirus-encoded miRNA expression in healthy and inflamed gingiva in obese and non-obese human subjects.

Principal findings:

Expression of numerous human miRNAs and three candidate viral miRNAs were altered in diseased gingiva. Mechanistically, we show that expression of novel gene targets of miR-24 and miR-27a are altered in disease.

Practical implications:

Epigenetic changes mediated by disease-associated miRNAs (both human and virus) may regulate periodontal inflammation. Combinatorial targeting of human and herpesvirus-derived miRNAs may provide an emerging novel diagnostic molecular tool for periodontal inflammation.

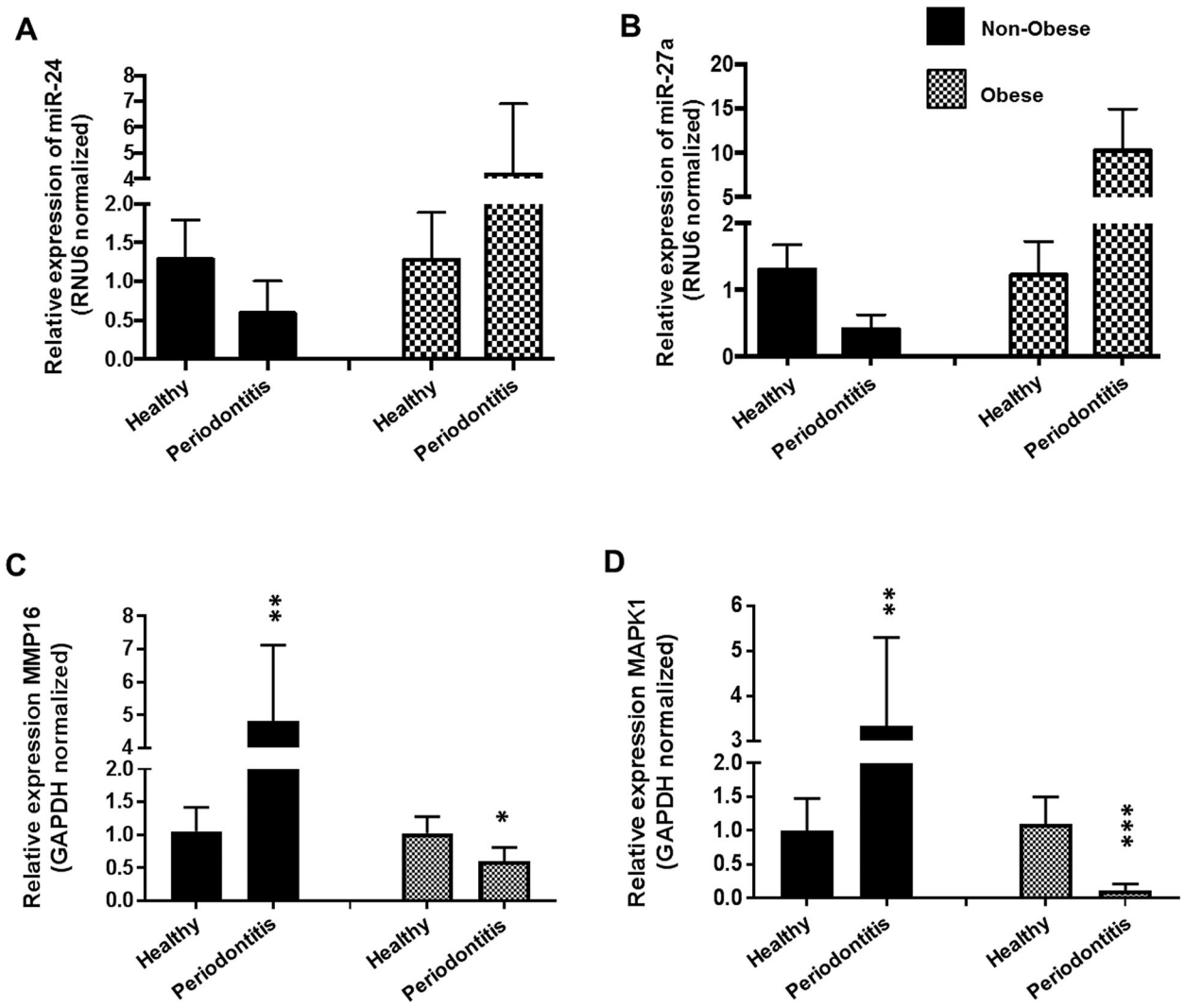

Figure 1.

MicroRNA and their predicted gene targets exhibit antagonistic expression pattern in healthy and periodontitis obese and non-obese subjects. Quantitative RT-PCR analysis of microRNA (miR-24 and miR-27a) and their predicted gene target (MMP-16 and MAPK1) expression in gingival biopsies of healthy and diseased obese and non-obese subjects (N=10). Histograms showing relative fold change in the expression of (A) miR-24, (B) miR-27a, (C) MMP-16 and (D) MAPK1. RNU6 and GAPDH was used as an endogenous control for miRNA and mRNA, respectively. Values are presented as mean ±SD. * p value < 0.05; **p<0.01; ***p<0.001; Student's t-test.

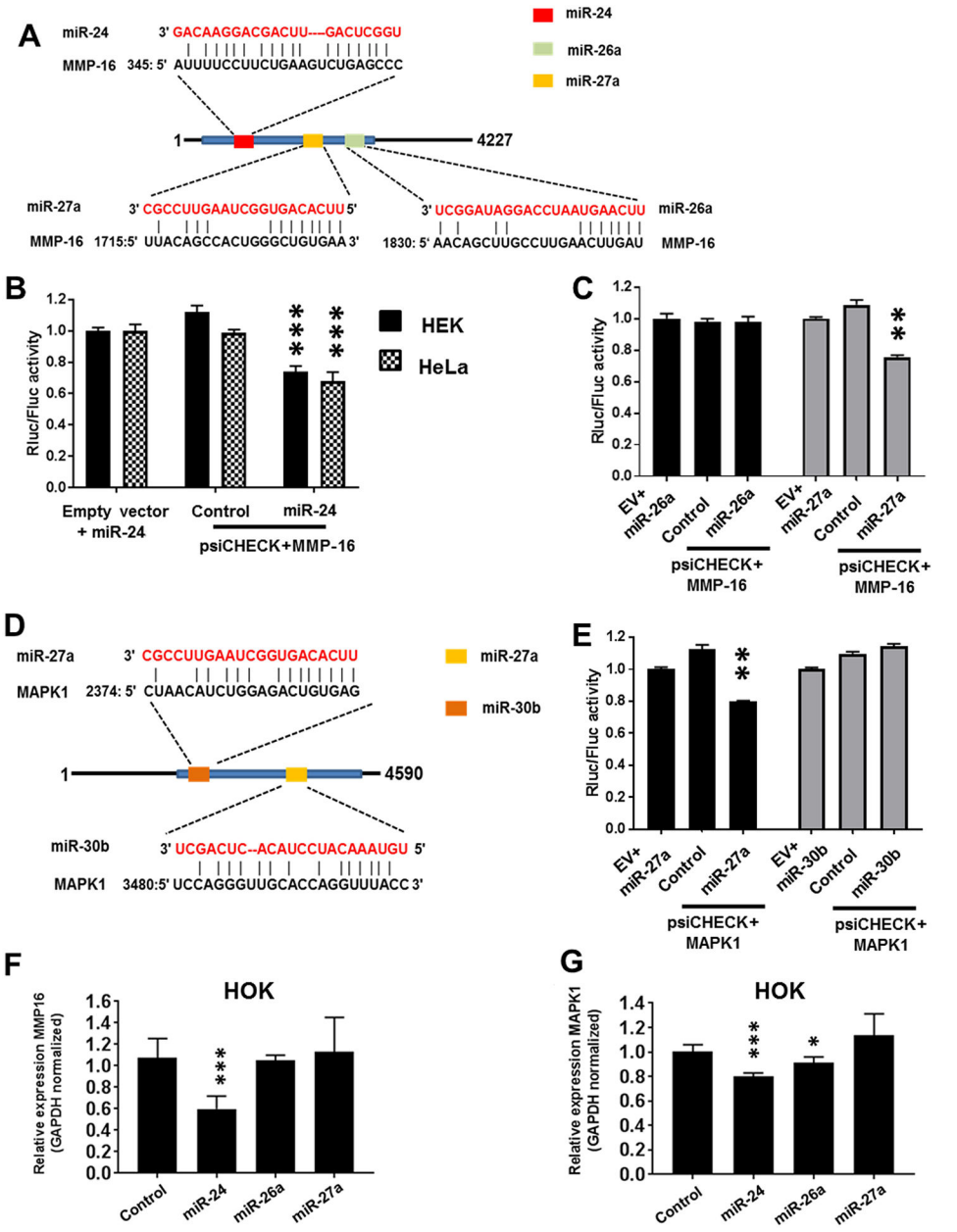

Figure 2.

MicroRNAs altered in gingival biopsies from periodontitis subjects target genes linked to tissue homeostasis and inflammation. (A) Sequence alignment of predicted miR-24 (red box), miR-26a (green box) and miR-27a (yellow box) binding sites in the 3′UTR of human MMP-16. Blue line represents the cloned part of the entire MMP-16 3′UTR. (B) HEK293 or HeLa cells were co-transfected with MMP-16 3′UTR construct and with miR-24 or control mimic. As a control, cells were co-transfected with empty psiCHECK-2 vector and miR-26a. Renilla activity was normalized to firefly activity, and the ratios subsequently normalized to empty vector transfected with miR-26a was set as 1. Data are expressed as ±SEM of four independent transfections. (C) HEK293 cells were co-transfected with MMP-16 3′UTR construct and with miR-26a, miR-27a or control mimic. As a control, cells

were co-transfected with empty psiCHECK-2 vector and miR-26a. Renilla activity was normalized to firefly activity, and the ratios subsequently normalized to empty vector transfected with miR-26a was set as 1. Data are expressed as ±SEM of four independent transfections. (D) Sequence alignment of predicted miR-27a (yellow box) and miR-27a (orange box) binding sites in the 3′UTR of human MAPK1. Blue line represents the cloned part of the entire MMP-16 3′UTR. (E) HEK293 cells were co-transfected with MAPK1 3′UTR construct and with miR-27a, miR-30b or control mimic. As a control, cells were co-transfected with empty psiCHECK-2 vector and miR-26a. Renilla activity was normalized to firefly activity, and the ratios subsequently normalized to empty vector transfected with miR-26a was set as 1. Data are expressed as ±SEM of four independent transfections. Quantitative RT-PCR analysis of MMP-16 and MAPK1 expression in miRNA or control mimic transfected human oral keratinocytes. Histograms showing relative fold change in the expression of (F) MMP-16 and (G) MAPK1. GAPDH was used as endogenous control. Values are presented as mean ±SD from three independent experiments. * p value < 0.05; **p<0.01; ***p<0.001; Student’s t-test.

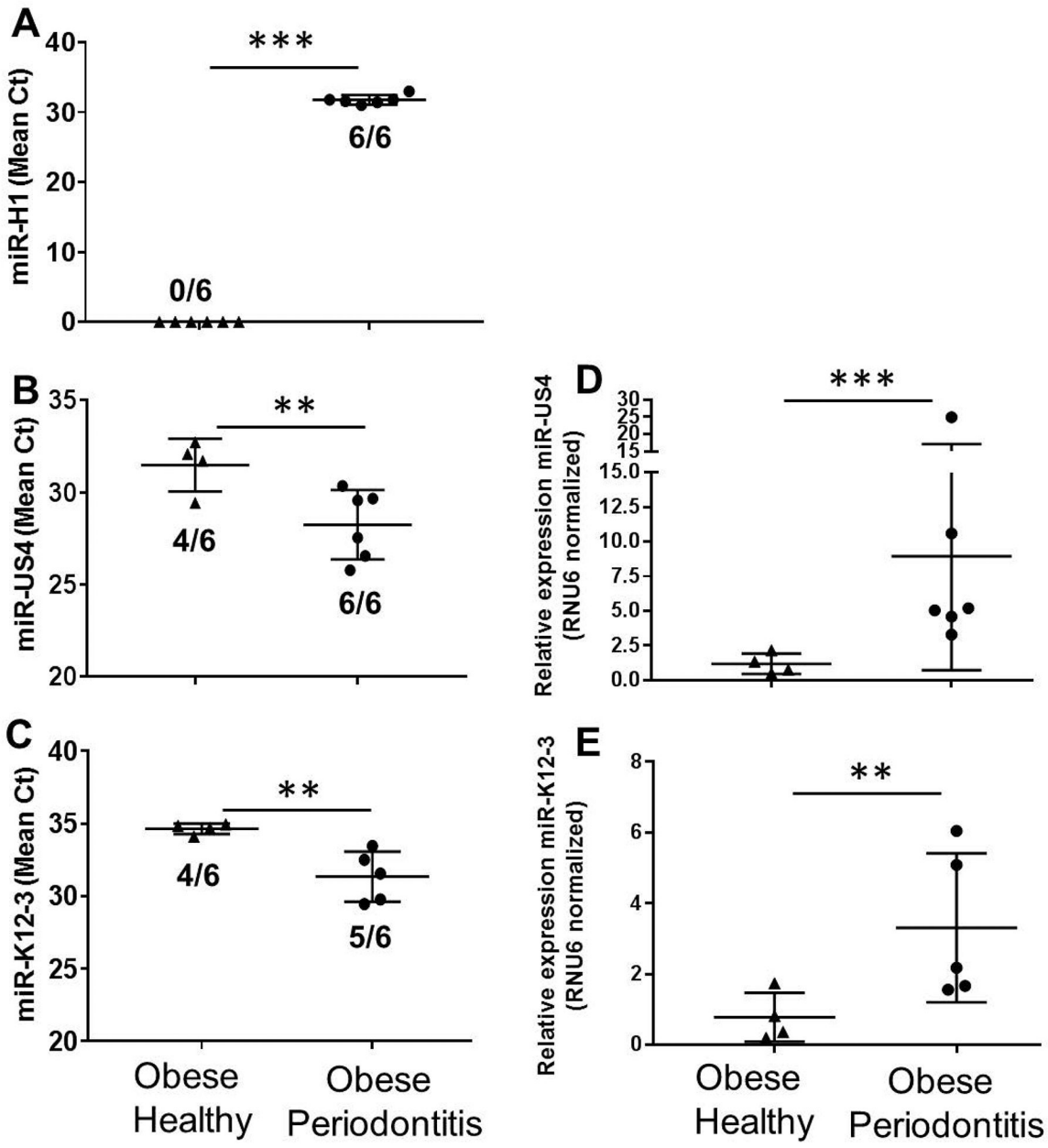

Figure 3.

Expression analysis of candidate viral miRNAs in healthy and diseased gingival samples. Total RNA was isolated from healthy and diseased tissues (n=6). Expression of three viral miRNAs miR-H1, miR-K12–3 and miR-US4 were detected by quantitative RT-PCR. Scatter plots show mean Ct value of (A) miR-H1, (B) miR-K12–3 and (C) miR-US4 in healthy and diseased gingiva. Numbers of positive samples are mentioned for each group. Relative expression of (D) miR-US4 and (E) miR-K12–3 was calculated after normalization with RNU6B as control. Student's t-test was used to calculate p-values. **p<0.01; ***p<0.001.

Table 1.

List of differentially expressed miRNA in diseased gingival biopsies derived from obese subjects compared with obese periodontally healthy specimens.

miRNA	Fold Change	p value
hsa-miR-21-5p	13.7924	0.001141
hsa-let-7f-5p	10.7559	0.000835
hsa-miR-29b-3p	10.2039	0.001421
hsa-miR-7-5p	9.5951	0.000011
hsa-miR-29c-3p	8.455	0.00144
hsa-miR-19a-3p	7.9754	0.003245
hsa-let-7g-5p	7.7104	0.000621
hsa-miR-374a-5p	6.9405	0.002386
hsa-miR-27a-3p	6.4735	0.00082
hsa-miR-146a-5p	6.2725	0.013057
hsa-miR-26b-5p	6.0369	0.002023
hsa-miR-27b-3p	5.7739	0.00103
hsa-miR-424-5p	5.489	0.048809
hsa-miR-126-3p	4.6583	0.000489
hsa-miR-101-3p	4.5711	0.000268
hsa-miR-20a-5p	4.5012	0.00284
hsa-let-7a-5p	4.2099	0.000984
hsa-miR-143-3p	4.1048	0.032395
hsa-miR-142-3p	4.0266	0.009795
hsa-let-7e-5p	3.9677	0.000765
hsa-miR-16-5p	3.9369	0.001541
hsa-miR-19b-3p	3.7798	0.030693
hsa-miR-17-5p	3.6969	0.003143
hsa-miR-195-5p	3.6101	0.005307
hsa-miR-15a-5p	3.2177	0.00912
hsa-let-7i-5p	3.1314	0.000321
hsa-let-7c-5p	3.0974	0.000536
hsa-miR-29a-3p	3.0765	0.000188
hsa-miR-93-5p	3.0606	0.001611
hsa-miR-26a-5p	2.9862	0.003244
hsa-let-7d-5p	2.9096	0.002102
hsa-miR-32-5p	2.8226	0.035062
hsa-miR-141-3p	2.778	0.028977
hsa-miR-28-5p	2.3219	0.047771
hsa-miR-24-3p	2.3139	0.0337
hsa-miR-155-5p	2.1083	0.027938

miRNA	Fold Change	p value
hsa-miR-30b-5p	2.0584	0.011846
hsa-miR-181b-5p	2.0294	0.01487
hsa-miR-30c-5p	1.676	0.041242
hsa-miR-423-5p	0.5111	0.016361
hsa-miR-196b-5p	0.3621	0.028421

Table 2.

List of differentially expressed miRNA in non-obese diseased gingiva compared with healthy tissues.

miRNA	Fold Change	p value
hsa-miR-150-5p	2.9668	0.018342
hsa-miR-146a-5p	0.4492	0.019474
hsa-miR-25-3p	0.4095	0.050268
hsa-miR-151a-5p	0.4034	0.040139
hsa-let-7d-5p	0.3653	0.015578
hsa-miR-17-5p	0.3	0.013462
hsa-miR-7-5p	0.2987	0.016641
hsa-miR-28-5p	0.2761	0.002401
hsa-miR-23b-3p	0.2723	0.014155
hsa-miR-185-5p	0.2718	0.037233
hsa-let-7g-5p	0.2612	0.028876
hsa-miR-24-3p	0.2603	0.010041
hsa-miR-374a-5p	0.2511	0.008054
hsa-let-7a-5p	0.2167	0.002435
hsa-miR-126-3p	0.2061	0.023744
hsa-let-7e-5p	0.2026	0.004664
hsa-miR-26a-5p	0.1954	0.002896
hsa-let-7f-5p	0.1607	0.004375
hsa-miR-27b-3p	0.1581	0.0011
hsa-miR-26b-5p	0.1384	0.006414
hsa-miR-21-5p	0.1054	0.003278

Table 3.

List of key pathways potentially affected by dysregulated miRNAs in obese and non-obese periodontitis subjects.

Common Pathway	FDR p- value	Number of genes	Number of miRNAs
ECM-receptor interaction	4.53E-09	65	40
Ubiquitin mediated proteolysis	2.44E-08	120	40
TGF-beta signaling pathway	2.15E-07	71	40
Bacterial invasion of epithelial cells	3.06E-06	67	40
FoxO signaling pathway	8.64E-06	114	40
PI3K-Akt signaling pathway	0.000148227	247	40
p53 signaling pathway	0.000178646	61	40
TNF signaling pathway	0.000436295	90	40
Apoptosis	0.002348869	73	40
Unique Pathway	**FDR p- value**	**Number of genes**	**Number of miRNAs**
Ribosome	1.18733E-05	114	40
Steroid Biosynthesis	0.000140212	18	35
Biosynthesis of unsaturated fatty acids	0.000529566	18	37
Thyroid Cancer	0.000529566	26	40
Toxoplasmosis	0.002493282	94	40
Vitamin B6 metabolism	0.006819932	6	19
Progesterone-mediated oocyte maturation	0.006819932	71	40
Circadian rhythm	0.010246299	27	40
Terpenoid backbone biosynthesis	0.037478314	18	37
Fatty acid degradation	0.042232691	32	32
Protein export	0.049958716	20	36