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ABSTRACT

The aim of this pilot investigation was to determine if microRNA expression differed in the presence or absence of obesity, comparing gingival biopsies obtained from patients with or without periodontal disease. Total RNA was extracted from gingival biopsy samples collected from 20 patients: 10 non-obese patients (BMI < 30 kg/m²) and 10 obese patients (BMI > 30 kg/m²), each group with 5 periodontally healthy sites and 5 chronic periodontitis sites. MicroRNA expression patterns were assessed with a quantitative microRNA PCR array to survey 88 candidate microRNA species. Four microRNA databases were used to identify potential relevant mRNA target genes of differentially expressed microRNAs. Two microRNA species (miR-18a, miR-30e) were up-regulated among obese individuals with a healthy periodontium. Two microRNA species (miR-30e, miR-106b) were up-regulated in non-obese individuals with periodontal disease. In the presence of periodontal disease and obesity, 9 of 11 listed microRNAs were significantly up-regulated (miR-15a, miR-18a, miR-22, miR-30d, miR-30e, miR-103, miR-106b, miR-130a, miR-142-3p, miR-185, and miR-210). Predicted targets include 69 different mRNAs from genes that comprise cytokines, chemokines, specific collagens, and regulators of glucose and lipid metabolism. The expression of specific microRNA species in obesity, which could also target and post-transcriptionally modulate cytokine mRNA, provides new insight into possible mechanisms of how risk factors might modify periodontal inflammation and may represent novel therapeutic targets.

KEY WORDS: microRNA, obesity, periodontitis, inflammation, epigenetics, PCR-array.

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MicroRNA Modulation in Obesity and Periodontitis

INTRODUCTION

Specific genetic polymorphisms have been associated with a susceptibility to gain weight (Adair and Prentice, 2004). Expression of these susceptibility genes can be influenced by epigenetic and environmental factors (Emilsson *et al.*, 2008). Transcription factors and microRNAs (miRNAs) are abundant regulatory systems that are modified by nutritional status. Evidence from *in vitro* studies and, more recently, from human studies has identified specific miRNAs that are associated with obesity. miRNAs are small, non-coding RNA molecules that bind and form complexes with mRNA species, usually to the 3'UTR of the mRNA. miRNA binding to mRNA, even with only moderate strand complementarity, serves to repress mRNA translation or can result in mRNA degradation. This lack of stringency can result in one miRNA affecting the translation of many different target genes. Thus, miRNA expression is a common translational feedback control mechanism that has been shown to modulate lipid and carbohydrate metabolism, as well as inflammatory pathways (Emilsson *et al.*, 2008; Iliopoulos *et al.*, 2008). It is possible that miRNAs that are induced by chronic nutritional stress leading to obesity may also non-parsimoniously modulate inflammatory pathways within periodontal tissues and affect disease expression.

The role of miRNAs in regulating adipocyte development and lipid metabolism has been well-established. Expression of miR-27 suppresses the translation of 2 very important early regulators of adipogenesis, namely, peroxisome proliferator-activated receptor- γ (PPAR γ) and CCAAT/enhancer binding protein α (Lin *et al.*, 2009). miR-143 expression is up-regulated 3.3-fold in obese mice, with similar increases in adipocyte differentiation markers PPAR γ and adipocyte fatty-acid-binding protein (Takanabe *et al.*, 2008). miR-122 was determined to be an important regulator of cholesterol and fatty-acid metabolism (Esau *et al.*, 2006). miRNA expression in human adipose tissue was first studied by Klötting *et al.* (2009), and significant correlations were found between expression of miRNA-17-5p, -132, -99a, -134, 181a, -145, and -197 and adipose tissue morphology, metabolic parameters (*i.e.*, HbA1c, fasting plasma glucose) leptin, adiponectin, and interleukin (IL)-6 levels. More recently, Martinelli *et al.* (2010) demonstrated that miR-519d was up-regulated in an obese population, and its predicted target was PPAR α .

Obesity is an established risk factor for periodontitis (Saito *et al.*, 2001, 2005; Al-Zahrani *et al.*, 2003; Wood *et al.*, 2003; Lundin *et al.*, 2004; Dalla Vecchia *et al.*, 2005; Genco *et al.*, 2005; Nishida *et al.*, 2005), but our understanding of how obesity modifies periodontal disease pathogenesis at the molecular level is unknown. Recently, some of these miRNA species have also been identified as possible modifiers of inflammatory pathways (Emilsson *et al.*, 2008; Iliopoulos *et al.*, 2008). For example, inhibition of miR-22 caused up-regulation of PPAR α and bone morphogenetic protein 7 (BMP7), blocked the inflammatory process through inhibition of IL-1B, and promoted repair in osteoarthritic chondrocytes (Iliopoulos *et al.*, 2008).

Currently, there are no human data to indicate whether miRNA species are differentially expressed in periodontally diseased tissues or whether obesity can modify miRNA expression. The overall goal of this study was to conduct a pilot investigation to determine whether obesity or periodontal disease modified miRNA expression, and whether there was any potential interaction between obesity and periodontitis that could involve miRNA modulation. We hypothesized that obesity may alter miRNA patterns and that some of these miRNA species would also be associated with inflammatory gene targets such as PPAR γ , nuclear factor kappa-B (NF κ B), and IL-6.

MATERIALS & METHODS

Study Participant Selection and Gingival Tissue Biopsies

This study was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. Twenty-four participants, aged 18 to 65 yrs, provided informed consent and were consecutively recruited from the clinics at the University of North Carolina School of Dentistry from May 2010 to January 2011. Demographic information and a thorough medical history questionnaire were obtained for all recruited individuals. Potential participants were excluded: if they had a chronic disease which could present with oral manifestations; if they had been treated with antibiotics for any medical or dental condition within 1 mo prior to the screening examination; if they were being chronically treated (*i.e.*, 2 wks or more) with any medication known to affect periodontal status (*e.g.*, phenytoin, calcium antagonists, cyclosporin, coumadin, non-steroidal anti-inflammatory drugs, aspirin > 81 mg) within 1 mo of the screening examination; if they had clinically significant organ disease, including impaired renal function, or any bleeding disorder; and if they had active infectious diseases such as hepatitis, HIV, or tuberculosis. Experienced study personnel recorded all recruited individuals' height and weight as measured with a precision stadiometer and a regularly calibrated digital scale, to compute Body Mass Index (BMI). Comprehensive periodontal examinations were performed on all participants by one of two calibrated examiners, and probing depths (PDs), clinical attachment level, and bleeding on probing (BOP) were recorded at 6 sites *per* tooth. Based on this information, a periodontal diagnosis was made according to the currently accepted American Academy of Periodontology 1999 Classification System of Periodontal Diseases and Conditions (Armitage, 1999). Potential participants were excluded if they had any acute or chronic diseases which resulted in oral manifestations or if they were taking medications known to affect the periodontium. Participants were enrolled into four groups (six/group) based on BMI (non-obese BMI < 30 kg/m² and obese BMI > 30 kg/m²) and the presence or absence of chronic periodontitis.

A single biopsy was taken from each participant. For healthy individuals, a gingival biopsy sample (including gingival epithelium, the col area, and the underlying connective tissue) was collected at the time of a scheduled crown-lengthening procedure. The biopsy site selected in healthy participants was at the site of a planned crown-lengthening procedure with healthy interproximal papillae. All healthy sites had a probing depth $\leq$ 4 mm, no bleeding on probing (BOP), and no evidence of

radiographic bone loss. A scalpel blade was used to obtain a biopsy with intrasulcular and inverse bevel incisions. The incision extended from the mid-palatal area of 2 adjacent teeth, beginning and ending intrasulcularly and going to the bone undermining the interproximal papillae, staying approximately 1 mm from the free gingival margin at the crest of the interproximal papillae and horizontally capturing the interproximal col area. For participants with chronic periodontitis, a gingival biopsy sample was obtained at the time of a scheduled periodontal flap procedure. A scalpel blade was used to obtain the sample from a severely inflamed interproximal periodontitis site, defined as a probing depth > 5 mm, and presence of BOP with evidence of radiographic bone loss. Gingival biopsy samples were immediately placed in RNeasy[®] (Applied Biosystems/Ambion Inc., Austin, TX, USA) overnight at 4°C, decanted, and stored at -80°C. Once collected chair-side, samples were placed in a cryovial that contained a bar-code label that was totally de-identified for laboratory processing, but facilitated tracking and linking to patient clinical information at the end of the laboratory processing for statistical analysis.

RNA Isolation and qRT-PCR

The study was conducted in a single masked manner such that all laboratory assessments were performed without knowledge of the participants' BMI or periodontal status. Total RNA, including small RNAs, was isolated from gingival tissue samples with a TissueLyser LT and miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was quantified by means of a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and quality and purity were assessed with the 2100 Bioanalyzer (Agilent, Foster City, CA, USA). Twenty samples (5/group based on BMI and periodontal status) were used for further analysis. Briefly, a volume of 600 ng total RNA was used for reverse-transcription reactions to generate complementary DNA (cDNA) with the RT² miRNA First Strand Kit (Qiagen, Frederick, MD, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed with 100 μ L of cDNA, according to the manufacturer's instructions (Qiagen), in a 7500 Sequence Detection System (ABI Prism, Applied Biosystems, Carlsbad, CA, USA). miRNA expression patterns were assessed with a human genome RT² miRNA PCR Array (SABiosciences) that contained probes for 88 of the most abundantly expressed and best-characterized miRNA sequences in the human miRNA genome. SNORD48 (Accession Number: NR_002745) and RNU6-2 (Accession Number: NR_002752) were used for normalization. Amplification parameters, conditions, and melting curves were set by the manufacturer. Data were analyzed by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), and results were reported as fold change in miRNA level, with a P-value < 0.05 considered significant. An ANOVA was used for overall group-effect significance and non-paired Student's *t* test for comparisons between groups without adjustment for multiple comparisons.

Bioinformatic miRNA Analysis and Target Selection

Predicted targets were selected for each of the statistically significant differentially expressed human miRNAs if they were

Table 1. Fold Difference in Expression of microRNA in Biopsy Samples by Obesity and Periodontal Status as Compared with Healthy Non-obese Biopsies and BMI Status

BMI Periodontal Status	Non-obese Periodontitis	Obese Periodontitis	Obese Healthy
MicroRNA			
miR-15a	1.6	2.7	1.8
miR-18a	1.3	1.5	1.5
miR-22	3.4	3.1	2.8
miR-30d	2.7	2.2	1.3
miR-30e	4.4	4.9	2.1
miR-103	1.0	1.5	1.2
miR-106b	4.9	6.4**	2.5
miR-130a	5.3	4.6	2.3
miR-142-3p	2.4	5.3**	1.9
miR-185	2.3	3.6	1.2
miR-210	1.4	2.3	1.2

†The referent group includes non-obese participants with a healthy periodontium. Highlighted values: $P < 0.05$, $**P \leq 0.01$.

Table 2. microRNAs Associated with GO Term Categories†

	Immunity & Inflammation	Collagen Metabolism	Lipid Metabolism	Carbohydrate Metabolism
miR-15a	X		X	
miR-18a	X	X		
miR-22	X		X	X
miR-30d	X	X		X
miR-30e	X	X	X	X
miR-103	X	X		X
miR-106b	X		X	
miR-130a	X	X	X	X
miR-142-3p	X		X	X
miR-185	X		X	
miR-210	X			

†More detail is provided in Appendix Table 2.

identified in either 2 or 3 out of 4 publically available miRNA databases, each of which uses different hybridization algorithms (see Appendix Table 1). Gene ontology (GO) terms associated with inflammation and collagen, glucose, and lipid metabolism were used to select target genes of interest.

RESULTS

Study Population Demographics

The participants' mean age was 45 yrs (± 13.3 SD), with no significant differences among the four groups in age, gender, and race (Appendix Table 2). Sixty percent of study participants were categorized as never-smokers, and 35% were ex-smokers. None of the participants had diabetes. Participants were not matched according to age, gender, or race. The non-obese participants had a mean BMI of 25.1 kg/m² (± 2.5 SD), and the mean BMI of the obese groups was 35.3 kg/m² (± 4.5 SD).

Differential miRNA Expression by BMI among Participants with Periodontitis

A comparison between participants with a healthy periodontium (and non-obese) and participants with periodontitis (either non-obese or obese) indicated that there was an up-regulation of 10 miRNAs ($P < 0.05$) (Fig. 1). Generally, the fold regulation varied from 1.5-fold to 6.4-fold. The majority of miRNA expressed in the periodontitis group reached statistical significance in those who were also obese. miR-30e was up-regulated in both groups, but it was statistically significant only in the periodontitis non-obese group. miR-106b reached a high level of statistical significance ($P < 0.01$) in the periodontitis participants regardless of their BMI classification, suggesting a significant role in periodontitis pathogenesis. Nevertheless, the fold regulation was greater (6.4-fold) for the participants who were obese compared with the non-obese participants (4.9-fold), consistent with an enhanced expression of several miRNA species in periodontitis

Figure 1. Fold regulation of miRNA species observed in periodontitis according to BMI status. Both obese and non-obese periodontitis groups were compared with the referent group (healthy periodontium, non-obese). miRNA species are listed if at least 1 of the pairwise comparisons was significant.

when obesity is present. In the presence of periodontitis or obesity, there was no significant down-regulation among the remaining 78 miRNA species surveyed. The effects of age, race, and gender were examined *post hoc* and were not found to be significant.

Differential miRNA Expression in Periodontitis among Obese Participants

Similarly, with a non-obese, periodontally healthy referent group, a comparison was made among obese individuals between those with or without periodontitis (Fig. 2). In periodontally healthy obese individuals, only 2 miRNA species (miR-18a, miR-30e) were significantly elevated. Although increased in both periodontal health and disease, miR-106b expression among obese individuals attained statistical significance only among those with periodontitis. Overall, 11 miRNAs were identified with significant up-regulation in this study cohort (Table 1). Nine of these were up-regulated and reached statistical significance only in periodontally diseased individuals who were obese. This suggests a role for miRNA in the interaction between obesity and periodontal inflammation.

Predicted mRNA Targets

To understand the potential modulatory role of these 11 up-regulated miRNA species associated with obesity and/or periodontal disease, we determined potential mRNA targets linked to immunology, inflammation, and metabolic disorders (as identified by GO Biological Process Terms) using 4 available miRNA databases. Sixty-nine different genes with miRNA target sequences were identified in 3 out of the 4 databases for the 11 miRNA species (Appendix Table 2A). When the targets validated in 2 out of 4 databases were included, 139 different genes were identified (listed below in brackets; Appendix Table 2B). The potential targets include key cytokines involved in systemic inflammation (Appendix Table 2): members of the interleukin family (IL1A, IL1F9, IL31 [IL15, IL17B, IL1F8, IL20]) and

Figure 2. Fold regulation of miRNA species observed in obese participants according to periodontal status compared with the referent non-obese participants (healthy periodontium). miRNA species are listed if at least 1 of the pairwise comparisons was significant.

receptors (IL13RA1, IL27RA [IL9R, IL10RB, IL1RL1, IL21R]), interferon family (IFNA16, IFNA21 [IFNA1, IFNA2, IFNK]) and receptors (IFNAR2, IRF2, IRF5), tumor necrosis factor superfamily (TNF, TNFAIP3, TNFSF8, TNFSF14 [TNFSF9]) and receptors (TNFRSF10A, TNFRSF10D, TNFRSF13B [TNFRSF13C]), and a suppressor of cytokine signaling (SOCS1); chemokines [CCL1, CCL5, CCL19]; proteins involved in collagen formation and fibroblast growth factor (COL9A3, COL13A, COL13A1, FGF21); receptor molecules involved in metabolism (IRS1, PEX3, PEX13, PPARG, PPARGC1B [PPARD, LRP1B, LRP2, LRP6, LRP8, VLDLR]); adhesion molecule (ICAM4); and proteases or molecules associated with bone metabolism (BAMBI, CASP6, MMP21 [MMP3, MMP10, MMP13, MMP16, OSTF1]).

DISCUSSION

The data are consistent with the concept that miRNAs induced by chronic nutritional stress leading to obesity may also non-parsimoniously modulate inflammatory pathways within periodontal tissues and affect disease expression. It is well-established that obesity leads to numerous morbidities such as cardiovascular and cerebrovascular disease and is associated with increased mortality from cardiovascular events and malignancies. As an impetus to discovering new therapeutic targets for adipogenesis, new discoveries have been made regarding certain miRNAs and their central role in adipocyte differentiation, metabolic functions, and insulin resistance (Heneghan et al., 2010). Recently, Nahid and colleagues (Nahid et al., 2011) have reported increased miR-146a expression in an experimental polymicrobial periodontitis model in mice, but no human reports exist. Therefore, the effect of miRNA transcriptional repression or mRNA cleavage on periodontal pathogenesis,

especially in the presence of obesity, is largely unknown. In this report, we provide the first human data implicating the role of miRNA as a potential mechanistic link between obesity and periodontal disease pathogenesis.

It is interesting to note that those differentially expressed miRNA species exhibited predicted targets that related predominantly to GO terms associated with key common and convergent molecular pathways (Table 2). These include inflammatory cytokines, immune regulation, bone, lipid, and glucose metabolism. This suggests that the metabolic influence of obesity, which increases the expression of specific miRNAs known to regulate lipid and glucose metabolism, also has mRNA targets that potentially modulate inflammation and connective tissue metabolism. For example, we found an up-regulation of miR-30e in the obese periodontally healthy patient group. Klöting *et al.* (2009) reported an increased expression of miR-30e in subcutaneous adipose tissue and suggested its potential involvement in the association between adipose tissue dysfunction and the development of obesity-related disorders like non-insulin-dependent diabetes mellitus. In this investigation, higher levels of miR-30e were also seen in the non-obese and periodontally diseased subset of patients, suggesting that inflammation also up-regulated this miRNA species. While none of the study participants had diabetes, a predicted target for miR-30e was IRS1, an insulin receptor that, when suppressed, is associated with insulin resistance and hyperglycemia. Thus, inflammatory-induced miRNA may potentially modulate local tissue metabolism.

Up-regulation of miR-103 was observed in participants with periodontal disease and an obese BMI. An up-regulation of miR-103 (Xie *et al.*, 2009) and miR-143 (Esau *et al.*, 2004; Kajimoto *et al.*, 2006) was observed during adipogenesis. We demonstrate that predicted targets for miR-103 include members of complement-related genes [C1QB, C1QL3], growth factors [FGF12, FGFR2, FIGF], interferon [IFNK], interleukins [IL1F8, IL20], and insulin-like peptide INSL5.

In a similar, but more comprehensive investigation, Iliopoulos and colleagues (2008) demonstrated an interesting association between arthritis and BMI. Human osteoarthritic cartilage was obtained and compared with normal cartilage. Among the most important differences, specific miRNAs (miR-22, miR-103) and proteins (PPARA, BMP7, IL1B) were highly correlated with BMI. Our study also found a significant up-regulation in miR-22 expression in the obese and periodontally diseased group compared with healthy/non-obese control individuals. The bioinformatic analysis used in this investigation did not reveal the identical predicted targets reported by Iliopoulos and colleagues. However, we identified several mRNA targets related to metabolic pathways (APOA5, IGFBP4) and inflammation (IL13RA1, IL1F9, IL27RA, IL31, IRF5, TNFRSF10D, TNFRSF13B) (Appendix Table 2).

Limitations of this study include the small sample size and the survey miRNA qPCR design that targeted only selected miRNA species. For example, although many were elevated (e.g., miR-18a and miR-30e), no different miRNAs were found to achieve statistical significance in non-obese, periodontally diseased individuals, reflecting a probable type 2 error. Obesity

appeared to further increase the expression of these miRNA species to achieve statistical significance. Clearly, additional studies with larger numbers of individuals will be needed to adequately assess the role of miRNA in regulating periodontal pathogenesis and the impact of obesity as a modifier. The increased expression of miR-106b was shared, with the periodontally diseased and obese group showing the largest up-regulation (6.4-fold). miR-106b is related to carcinogenesis and cell-cycle regulation (Ivanovska *et al.*, 2008; Li *et al.*, 2009). In this report, we demonstrate that miR-106b *in silico* predicted targets include genes related to inflammation and bone metabolism pathways, such as prostaglandin E₂ receptor (PTGER3) and tumor necrosis factor superfamily members (TNFSF14, TNFRSF10A) (Appendix Table 2). PGE₂ and TNF are established markers of gingival inflammation within gingival crevicular fluid (Andriankaja *et al.*, 2009). It is important to emphasize that these *in silico* target findings reflect theoretical targets calculated from sequence complementarity and secondary structure predictions. These hypothetical targets will require confirmation by functional assays, such as the use of a luciferase reporter construct to assess, for instance, 3'UTR mRNA targeting PGE₂ or TNF. Further confirmation would require *in vitro* over-expression, knockdown studies, and knockout mice to validate these findings.

In conclusion, this investigation demonstrates that the comorbidities of obesity and periodontitis are associated with significant local up-regulation of several miRNA species that share inflammatory and metabolic mRNA targets. The generalizability of these conclusions is dampened by the small sample size, the limited number of survey miRNA species, and the non-correction for multiple comparisons in the analyses. However, the consistency of the trends and specificity of the response provide further support for the findings. This is a proof-of-concept report, and larger studies will be needed to further validate these findings. It is enticing, however, that miRNAs identified in this study may play a crucial role in linking the underlying molecular pathways between obesity and periodontal inflammation. Furthermore, these miRNA species may represent novel therapeutic targets to manage periodontal disease.

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