**Association between serum miRNAs and gingival gene expression in an obese rat model**

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## Abstract

### Introduction

Recent studies have reported a relationship between periodontitis and obesity; however, the mechanisms of obesity's effects on periodontitis are not well understood. On the other hand, microRNAs (miRNAs) are known to play key roles in the post-transcriptional regulation gene expression by suppressing translation and protein synthesis. We examined the association between obesity-related miRNAs and gene expression in gingival tissue using miRNA-messenger RNA (mRNA) pairing analysis in an obese rat model.

### Methods

Sixteen male Wistar rats aged 8 weeks old were divided into two groups; the control group was fed a normal powdered food for 8 weeks, and the obesity group was fed a high-fat diet for 8 weeks. Distance from the cement-enamel junction to the alveolar bone crest of the first molars were measured. miRNA microarray analysis was performed on samples

of serum and gingival tissue; the resulting data were used to calculate fold-changes in miRNA levels in the obesity group relative to the control group, and miRNA-mRNA pairing analysis was performed to identify mRNAs potentially targeted by miRNAs of interest.

**Results**

Alveolar bone loss in the obesity group exceeded that in the control group ($P = 0.017$). miRNA-mRNA pairing analysis identified an association between 4 miRNAs (*miR-759*, *miR-9a-3p*, *miR-203b-3p*, and *miR-878*) that were differentially expressed in the obesity and control groups and 7 genes (*Ly86*, *Arid5b*, *Rgs18*, *Mlana*, *P2ry13*, *Kif1b*, and *Myt1*) expressed in gingival tissue.

**Conclusion**

This study revealed that several miRNAs play an important role in the mechanism of periodontal disease progression induced by the obesity.

**Keywords**

periodontitis, obesity, microRNA, mRNA, experimental animal model

## 1. INTRODUCTION

Obesity is defined as a systemic disease in which abnormal or excessive body fat accumulation adversely affects health.[1] Obesity is a major public health problem in developed and developing countries. As of 2016, the World Health Organization estimated that 39% of the adult population worldwide are overweight, and that 13% are obese.[2] Obesity is associated with low-grade chronic inflammation and has been shown to predispose to many systemic diseases, including atherosclerosis, cardiovascular disease, hypertension, and diabetes.[3] The development of obesity has been reported to promote increases in the circulating levels of inflammatory proteins secreted from adipocytes and adipose tissue-derived macrophages, leading to systemic inflammation.[4]

Periodontitis, which is a chronic inflammation of the supporting structures of the tooth, is one of the most common chronic diseases in the world.[5,6] Recent studies have reported a relationship between periodontitis and systemic disease such as diabetes, metabolic syndrome, rheumatoid arthritis, and cardiovascular diseases.[7] Several epidemiological studies have reported that obesity is a risk factor for periodontitis.[8,9] Animal and biological research has reported associations between periodontitis and obesity.[10,11] Those studies revealed that obesity is a chronic source of inflammatory cytokines or oxidative stress, factors that may affect periodontal tissues. However, the

mechanisms of obesity's effects on periodontitis are not well understood. Other circulating mediators may play important roles in the progression of periodontal disease following obesity.

MicroRNAs (miRNAs) are single-stranded non-coding RNAs, approximately 21-23 nucleotides in length, that play key roles in the post-transcriptional regulation of gene expression, acting by suppressing phases of translation and protein synthesis.[12,13] These small molecules are known to bind to the three-prime untranslated regions (3'-UTRs) of targeted messenger RNAs (mRNAs) via sequence complementarity.[14] MiRNAs have been associated with the pathogenesis of various diseases, including obesity and diabetes.[15] Especially in the pathology of obesity, numerous miRNAs are involved in the inflammation and intercellular communications in the local tissues.[16] Therefore, we hypothesized that miRNAs that are produced by tissues of obese animals may participate in the regulation of mRNAs, affecting associated functions, in the periodontal tissues of these organisms. Specifically, we postulated that obesity may alter the serum miRNA profile, and that some of these miRNAs may be associated with changes in the expression in periodontal tissue of genes associated with inflammatory functions. The purpose of the present study was to examine the association between microRNAs related to obesity and gene expression in periodontal tissue using miRNA-mRNA pairing analysis in a high-

cholesterol-diet-fed rat model.

## 2. MATERIALS AND METHODS

### 2.1 Animals

Sixteen male Wistar rats (Charles River Laboratories Japan Inc., Yokohama, Japan), aged 8 weeks old at study start, were used in this study. Animals were housed in an air-conditioned room (22-25°C) with 12-h/12-h light/dark cycle. All rats received food and water *ad libitum*. The experimental procedures were approved by the Animal Care and Use Committee, Okayama University (Approval No. OKU-2017418). The present study was performed in accordance with the ARRIVE guidelines.

### 2.2 Experimental design

Rats were randomized and assigned to two groups. During the experimental period, the control group (n = 8) was fed a normal powdered food (MF, Oriental Yeast Co., Ltd., Osaka, Japan) containing 55.3% carbohydrate, 23.1% protein, and 5.1% fat for 8 weeks. The obesity group (n = 8) was fed a high-fat food (F2HFD2, Oriental Yeast Co., Ltd., Osaka, Japan) containing 60.0% fat, 24.5% protein, and 7.5% carbohydrate for 8 weeks.[17]

After the experimental period, the rats were euthanized under anesthesia with diethyl

ether. Blood samples (no anti-coagulant) were collected from the heart at euthanasia; following clotting, the samples were subjected to centrifugation at 1,500 × g for 15 min and the resulting sera were transferred to separate tubes. At necropsy, the maxillary molar regions and the right mandibular regions were resected *en bloc* from each rat. The following analyses were performed using these samples.

**2.3 Serum analyses**

To evaluate the degree of obesity, aliquots (100 μL/animal) of serum were used to determine the levels of serum triglycerides, total cholesterol, and low- (LDL) and high- (HDL) density lipoprotein cholesterol using a gel permeation high-performance liquid chromatography system (Skylight Biotech, Inc., Akita, Japan).[18]

**2.4 Measurements of alveolar bone loss**

The right mandibular regions were de-fleshed using curettes, stained with 1% aqueous methylene blue (Sigma, St. Louis, MO, USA) for five minutes, and photographed using a digital camera (Nikon Instruments, Inc., Tokyo, Japan). The distance (mm) from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC) of the first molars (three roots) was measured using the software (ImageJ, NIH, Bethesda, MD, USA). Alveolar

bone loss was defined as the mean distance from the CEJ to the ABC at three roots. [19, 20]

### 2.5 Total RNA extraction

Total RNA, including miRNAs, was extracted from serum samples (100 μL/animal) using the RNA extraction kit (3D-Gene RNA extraction reagent from the liquid sample kit, Toray Co., Ltd., Kanagawa, Japan). Separately, total RNA was extracted from gingival tissue (50 mg/animal) samples using a commercially available kit (RNeasy Plus Universal Kits, QIAGEN, Venlo, Netherlands). Extracted total RNA was quantified by the spectrophotometer (NanoDrop 2000, ThermoFisher Scientific, Inc., Waltham, MA, USA). The quality of the extracted total RNA was evaluated using the analyzer (Agilent 2100 bioanalyzer, Agilent Technologies, Santa Clara, CA, USA).

### 2.6 miRNA microarray in serum

miRNA microarray analysis in the serum samples was performed to identify miRNAs associated with obesity. Samples from obesity and control groups were divided into 4 groups each (obesity 1, n=2; obesity 2, n=2; obesity 3, n=2; obesity 4, n=2; control 1, n=2; control 2, n=2; control 3, n=2; and control 4, n=2) and pooled samples were prepared. RNA samples (0.5 μg/sample) were labeled using a kit (3D-Gene miRNA labeling kit,

Toray Co., Ltd., Kanagawa, Japan) and hybridized to miRNA chips (3D-Gene Rat miRNA Oligo chips, Toray Co., Ltd., Kanagawa, Japan) according to the manufacturer's instructions. The hybridized slides were scanned and analyzed using a scanner (3D-Gene Scanner 3000, Toray Co., Ltd., Kanagawa, Japan). The signal from each spot was normalized to the mean intensity of the background signals determined from the signal intensity of the blank spots.

**2.7 mRNA microarray in gingival tissue**

mRNA microarray analysis in the gingival tissue samples was performed to identify mRNAs associated with obesity. Samples from obesity and control groups were divided into 4 groups each in the same way as the miRNA microarray analysis and pooled samples were prepared. RNA samples (1 μg/sample) were amplified using a commercially available kit (Amino Allyl Message Amplification II Kit, ThermoFisher Scientific, Inc., Waltham, MA, USA). Amplified RNA was quantified by the spectrophotometer (NanoDrop 2000), labeled using a stainer (Amersham Cy5 Mono-Reactive Dye, GE Healthcare Life Sciences Japan, Tokyo, Japan), and hybridized to mRNA chips (3D-Gene Rat mRNA Oligo chips, Toray Co., Ltd., Kanagawa, Japan) according to the manufacturer's instructions. The hybridized slides were scanned and analyzed using a

scanner (3D-Gene Scanner 3000). The signal from each spot was normalized by the mean intensity of the background signals determined from the signal intensity of the blank spots.

### 2.8 Selection of miRNAs and mRNAs following microarray analysis

The signal intensity was calculated by subtracting background signal intensity from raw data. The global normalization method was used to normalize the background-subtracted signal intensities to the median of these signal intensities, such that the median was defined as 25 units. For each miRNA or mRNA, the fold change (FC) value of the obesity group relative to the control group was calculated.

The signal of miRNAs or mRNAs of the obesity group and the control group were compared by a two-tailed non-paired Student's *t*-test using a statistical software (SPSS 25.0J for Windows; IBM Japan, Tokyo, Japan). The statistical significance was set at $P < 0.05$. We then selected miRNAs and mRNAs according to their FC values. For miRNAs, an FC of 0.67 or 1.5 was used as a cut-off value. For mRNAs, an FC of $\geq 2$ or $\leq 0.5$ was considered indicative of differential expression of the gene.[21]

### 2.10 miRNA-mRNA pairing analysis

Web server database (TargetScan, TargetScanHuman, Release 7.2: March 2018,

http://www.targetscan.org/vert_72/) was used to predict the mRNAs targeted by miRNAs.[22] This database predicts biological targets of miRNAs by searching for the presence of conserved 8-mer, 7-mer, and 6-mer sites in the seed region of 3′-UTR of mRNAs.[23]

### 2.9 Validation of gingival mRNA expression

Quantitative real-time polymerase chain reaction (RT-qPCR) analysis was performed using an analyzer (Mx3000P Real-time QPCR System; Agilent Technologies). Analyses were performed using an assay kit (TaqMan Gene Expression Assays, ThermoFisher Scientific, Inc.). We used the 2-ΔΔCt method to analyze the relative quantification of mRNAs.[24] We normalized the relative expression of mRNA by log10 transformation for analysis. Statistical significance was calculated using the Mann-Whitney *U* test.

## 3. RESULTS

The mean ± SD body weight of rats in the obesity and control groups at 8 weeks of age (baseline) were 274.8 ± 12.7 g and 274.7 ± 11.9 g, respectively. At 16 weeks of age, the mean ± SD body weight of rats in the obesity group was significantly higher than that of control group (457.1 ± 32.2 g and 562.6 ± 40.6 g, respectively) ($P = 0.007$). Also at 16

weeks, serum triglycerides, total cholesterol, and LDL cholesterol in the obesity group were significantly higher than those in the control group ($P = 0.001$, <0.001, and 0.009, respectively). In contrast, the 16-week serum HDL cholesterol in the obesity group was lower than that in the control group, but this difference was not significant ($P = 0.313$) (Table 1).

Alveolar bone loss was significantly greater in the obesity group than in the control group ($P = 0.017$) (Table 2, Figure 1).

The microarray analysis detected a total of 65 miRNAs and 185 mRNA genes. Among 65 miRNAs, 8 showed significant changes, with 7 miRNAs up-regulated and 1 down-regulated in the obesity group compared to the control group (Table 3). Among 185 mRNA genes, 23 showed significant changes, with 5 mRNAs up-regulated and 18 down-regulated in the obesity group compared to the control group (Table 4).

miRNA-mRNA pairing analysis was performed to select mRNAs that might be targeted by the 8 serum miRNAs with altered levels. Among these target genes, seven (*Ly86*, *Arid5b*, *Rgs18*, *Mlana*, *P2ry13*, *Kif1b*, and *Myt1*) also showed significant changes in expression as assessed by our microarray analysis of mRNAs (Table 5).

Results of the RT-qPCR analysis showed that *Ly86*, *Arid5b*, *Rgs18*, *Mlana*, and *P2ry13* in gingival tissue were expressed significantly lower in the obesity group than in

the control group (Figure 2; A-E). Also, Kif1b and Myt1 in gingival tissue were expressed significantly higher in the obesity group than in the control group (Figure 2; F, G).

## 4. DISCUSSION

It has been reported that miRNAs from some organs may affect the circulating levels of miRNAs, thereby altering mRNA levels and/or expression in other, distant organs.[25] We investigated the relationship between serum miRNA and gingival mRNA profiles, as well as the influence of obesity on gingival tissue in a rat model of obesity. Our serum miRNA microarray analysis showed that 3 miRNAs (*miR-759*, *miR-9a-3p*, and *miR-203b-3p*) were up-regulated and one (*miR-878*) was down-regulated in obese rats compared to control animals. Gingival mRNA microarray analysis showed that 5 mRNAs (*Ly86*, *Arid5b*, *Rgs18*, *Mlana*, and *P2ry13*) were down-regulated and 2 mRNAs (*Kif1b* and *Myt1*) were up-regulated in obese rats compared to control animals. miRNA-mRNA pairing analysis predicted that the 4 serum miRNAs that showed differential accumulation may specifically target the 7 gingival mRNAs that showed differential accumulation. To the best of our knowledge, this research is the first study to show the association between the levels of serum miRNAs and gingival mRNAs in an obesity rat model.

Ly86, also named myeloid differentiation protein 1 (MD-1), is a well-known secreted

glycoprotein that targets Toll-like receptor (TLR) 4-mediated pathways.[26] *TLR4* is considered an important gene for periodontal disease because of its role in the immune system.[27] In a previous study, TLR4/Mitogen-activated Protein Kinase (MAPK)/Nuclear Factor kappa B (NF-κB) pathways were shown to be over-activated in *MD-1* knockout mice.[28] Also, application of lipopolysaccharide to human periodontal ligament cells has been shown to decrease the expression of Ly86.[29] Therefore, we postulate that serum *miR-759* accumulation is up-regulated, and gingival *Ly86* mRNA accumulation is down-regulated, in obese animals, resulting in the activation of the TLR4/MAPK/NF-κB pathways and induction of the production of inflammatory cytokines.

Rgs18 is a cytosolic protein belonging to a class designated "regulators of G-protein signaling" (RGS) proteins. RGS proteins are thought to play a central role in physiological regulation of the G-protein cycle, and the importance of RGS proteins in cell signaling has been demonstrated in various cells.[30,31] In a previous study, expression of Rgs18 was significantly inhibited by receptor activator of NF-κB ligand (RANKL) in a macrophage/monocyte lineage cell line and in bone marrow-derived monocytes.[32] Also, inhibition of Rgs18 (by stimulation with RANKL) has been shown to prominently enhance osteoclastogenesis.[32] Therefore, we postulate that *miR-759* accumulation is up-regulated, and gingival *Rgs18* mRNA accumulation is down-regulated, in obese animals,

affecting the acid-sensing osteoclastogenic G-protein signaling pathway and resulting in alveolar bone resorption.

Mlana, also known as melanoma antigen, is a specific marker of melanocytes and has been shown to be recognized by $T_{H1}$ cells.[33] However, not all melanomas express Mlana,[34] and a significant decrease in immunohistochemical staining for Mlana has been seen in metastatic lesions and in amelanotic variants.[35] On the other hand, animal and clinical studies have shown that obesity is associated with the progression of melanoma.[36,37] Although the detailed mechanism of these effects remains unknown, obesity may cause the development of melanoma in the gingiva, as inferred from the results of the present study.

P2ry13 is widely distributed in different tissues and cell types, including osteoclasts and osteoblasts.[38,39] In a previous study, deletion of the *P2ry13* gene led to a 40% reduction in trabecular bone mass, a 50% reduction in osteoblast and osteoclast numbers, and an overall 50% reduction in the rate of bone remodeling in mice.[40] Additionally, down-regulation of RhoA/ROCK I signaling and a decrease in the RANKL/osteoprotegerin ratio were observed in osteoblasts isolated from *P2ry13* knockout mice.[40] Therefore, we suggest that up-regulation of serum *miR-9a3p* and down-regulation of gingival *P2ry13* expression in obese animals may lead to alveolar bone

resorption through alterations in bone turnover rates.

We also observed that changes in the expression of other gingival genes such as *Arid5b*, *Kif1b*, and *Myt1* were significantly associated with obesity in our rat model. However, the effects and mechanisms of the changes in expression of these genes in periodontal tissue cannot be inferred, given the lack of literature information about these loci. Further studies will be needed to clarify the mechanism whereby these genes may effect changes in gingival tissue.

This study had some limitations. First, we did not investigate miRNA expression levels in adipose tissue. Therefore, it is unclear whether the observed changes in the serum miRNA profile actually reflect changes in adipose tissue. Second, we did not investigate miRNA expression levels in gingival tissue. So, it remains unclear whether obesity induced periodontitis via serum miRNA identified in the present study. Recent clinical study has reported an association between serum miRNAs and gingival crevicular fluid (GCF) miRNAs, and GCF miRNA reflects periodontal environment change.[41] Our previous study showed that several serum miRNAs were found in the periodontitis rat model.[42] Therefore, it is possible that obesity induced periodontitis firstly, and then gingival-derived miRNAs appeared in serum. Investigation of miRNA expression levels in gingival tissue will be needed to understand the effect of obesity on gingival tissue.

Third, we only performed miRNA-mRNA pairing analysis to determine the association between the levels of serum miRNAs and gingival mRNAs. The detailed roles of these miRNAs will need to be investigated further in future studies. Finally, all animals in this study were male. Different results may be obtained in female rats.

## 5. CONCLUSIONS

We demonstrated that serum miRNA and gingival mRNA profiles are altered in obese rats compared to control animals. We postulate that these miRNAs may play an important role in the progression of periodontal disease induced by the obesity.

## Author Contributions

TM contributed to conception, design, data acquisition and interpretation, performed all statistical analyses, drafted and critically revised the manuscript. TK, YS, and TY contributed to conception, design, data acquisition and interpretation, and critically revised the manuscript. DE and MM contributed to conception, design, and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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**Figure Legends**

Figure 1

Alveolar bone loss (red lines) (A: Control group, B: Obesity group).

Figure 2

Expression of *Ly86* (A) ($P$ = 0.019), *Arid5b* (B) ($P$ = 0.005), *Rgs18* (C) ($P$ = 0.048), *Mlana* (D) ($P$ = 0.016), *P2ry13* (E) ($P$ = 0.030), *Kif1b* (F) ($P$ = 0.030), and *Myt1* (G) ($P$ = 0.047) in gingival tissue assessed by quantitative real-time PCR. Data are expressed as the median, 25th and 75th percentiles for 4 pooled samples (2 rats each) each in the obesity and control groups. Statistical significance was calculated using the Mann-Whitney $U$ test.