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Comparison of inflammatory microRNA expression in healthy and periodontitis tissues

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Key words: microarray, real-time PCR, gingiva

ABSTRACT: MicroRNAs (miRNAs) are short RNA molecules that negatively regulate gene expression primarily by degrading target mRNA or inhibit the translation of protein product. Recently, many reports have shown the altered miRNA expression in various diseases. However, there are no reports on miRNA expression related to periodontitis. Thus, this study aimed to compare the miRNAs differentially expressed in healthy and chronic periodontitis tissues and to determine the miRNAs closely associated with chronic periodontitis. To find out the miRNAs differentially induced in healthy and chronic periodontitis tissues, miRNA microarray was carried out and the expression of miRNAs was confirmed by real-time PCR. According to miRNA microarray analyses, six miRNA genes, let-7a, let-7c, miR-130a, miR301a, miR-520d, and miR-548a, were up-regulated more than 8 fold compared to the healthy gingiva. The expression of twenty-two miRNAs was increased more than 4 fold. Among these miRNAs, eight miRNAs which are known to be closely related to inflammation were selected. Six of these miRNA genes, miR-181b, miR-19b, miR-23a, miR-30a, miR-let7a, and miR-301a, were amplified successfully and increased much more in periodontitis gingivae than in healthy ones. In summary, this study indicate that six miRNAs up-regulated in periodontitis gingiva may play a key role in chronic periodontitis.

Introduction

Periodontal disease involves inflammation of the periodontium and is accompanied by apical migration of the junctional epithelium, leading the destruction of connective tissue attachment and alveolar bone loss (Flemmig, 1999; Suzuki, 1988). Chronic periodontitis is the most common form of destructive periodontal disease (Flemmig, 1999) and shows a slow disease progression that is characterized by bursts of disease activity separated by quiescent periods of varying durations (Socransky *et al*., 1984). Periodontal diseases are infectious and initiated as a consequence of dental plaque biofilm formation (Socransky and Haffajee, 2002; Lindhe *et al*., 1975). The host immune defense against plaque bacteria plays an important role in the development periodontal disease (Kinane and Lappin, 2001). Therefore, plaque bacteria are essential for initiating and evoking the chronic inflammatory response in periodontal tissues (Byrne *et al*., 2009; Amano, 2010). At the same time, it was reported that destructive processes occurring as part of the host inflammatory response are responsible for periodontal tissue breakdown, leading to the clinical signs of periodontitis (Graves and Cochran, 2003; Taubman *et al*., 2005). Therefore, the characteristic features of chronic periodontitis occur mainly as a result of activation of the host-derived immune and inflammatory defense mechanism. As a gen-

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eral rule, host inflammatory mediators are associated with tissue destruction, whereas anti-inflammatory mediators counteract and attenuate disease progression (Kinane and Lappin, 2001).

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules, and a new key player in the cellular control of gene expression. MiRNA regulate gene expression post-transcriptionally by binding to the complementary sequences in the coding or 3' untranslated region of the target messenger RNAs (mRNAs), leading to either blocking translation or inducing target mRNA degradation. Therefore, miRNAs participate in regular biological processes within the cells and tissues, and are also involved in pathological processes (Bartel, 2009; Guarnieri and DiLeone, 2008). Dysregulation of these molecules is a hallmark of cancer (Shenouda and Alahari, 2009; Chang *et al*., 2007) and investigators are currently examining their role in the pathogenesis of inflammatory diseases (Oglesby *et al*., 2010; Matsushima *et al*., 2011). In particular, miR-146 and miR-155 have been reported to be related to inflammation and bacterial infections (Xiao *et al*., 2009; Liu *et al*., 2010). However, there are no reports related to periodontal disease. Considering that periodontal disease is an inflammatory disease resulting from an interaction between dental plaque bacteria and the host immune system, it was likely that a differential expression of inflammatory miRNA would occur in periodontitis tissue as compared to healthy gingival tissue.

Therefore, miRNA microarray experiments were carried out with healthy and chronic periodontitis gingival tissues to compare the miRNAs differentially expressed in healthy and chronic periodontitis tissues and to determine the miRNAs closely associated with chronic periodontitis.

Materials and Methods

Gingival samples

Normal healthy gingiva and diseased gingival tissues were obtained from patients who were scheduled to undergo periodontal treatment at the Department of Periodontics of Pusan National Dental School. The inclusion criteria were partially or fully dentate patients, who were healthy with no evidence of known systemic modifiers of periodontal disease (type 1 and 2 diabetes mellitus, osteoporosis, and medications known to affect periodontal tissues). Chronic periodontitis patients had moderate-to-advanced periodontal disease including probing depth, > 5 mm; attachment loss, > 3 mm; radiographic evidence of extensive bone loss. The gingival biopsies were obtained at the time of periodontal surgery. The control group consisted of subjects presenting clinically healthy gingival tissues (low scores of bleeding on probing under 10% of the sites; no sites with probing depth > 3 mm or presenting attachment loss), from which biopsies of the gingival tissue were taken during surgical procedures for crown lengthening. The experimental protocol used was approved by the Institutional Review Board of Pusan National University. Informed consent was obtained from all subjects before the study. The gingival tissue samples were stored at –70°C until analyzed. For PCR analysis, small RNA was isolated from the tissue samples that had been homogenized on ice.

MiRNA microarray

MiRNA expression profiling of the periodontitis tissue was examined using a RT^2 miRNA PCR array system (SABiosciences, Frederick, MD) according to the manufacturer's instructions. The total RNA was isolated from the tissue using a mirVana™ miRNA Isolation Kit according to the procedure provided by the manufacturer. The cDNA was prepared from 1 μg total RNA using a RT^2 PCR array first strand kit (SABiosciences, Frederick, MD). A total volume of 25 μl of a PCR mixture, which included 12.5 μl of RT^2 Real-Time SYBR Green/ROX PCR master mix from SABiosciences (containing HotStart DNA polymerase, SYBR Green dye and the ROX reference dye), 11.5 μl of H_2O, and 1 μl of template cDNA, was loaded in each well of a PCR array. PCR amplification was carried out with an initial 10 min step at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent signal from SYBR Green was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. The data was analyzed by RT^2 Profiler PCR array Data analysis (SABiosciences, Frederick, MD).

Real-time PCR

The differentially expressed miRNAs selected by microarray analysis were validated further by TaqMan miRNA assays (Applied Biosystems, Foster City, CA). The results were normalized to the level of RNU44 expression. The results of miRNA expression are re-

ported as the mean ± standard error of the mean. *P* values < 0.05 based on a Student's *t*-test were considered significant.

Results

Inflammatory miRNA is up-regulated in periodontitis tissue as compared to healthy gingiva

MiRNA microarray analysis including 93 human inflammatory miRNAs (Table 1) was carried out to identify miRNA whose expression changes in periodontitis gingiva. Figure 1 presents the miRNA microarray quality result. The microarray analysis quality was marked as OKAY, A, B, and C. In this microarray profiling, more than 70% of the miRNA genes were analyzed as OKAY, A and B, which could be included for further analyses in the microarray experiments.

According to miRNA microarray analyses (Table 2, Table 3) six miRNA genes (let-7a, let-7c, miR-130a, miR301a, miR-520d, and miR-548a), were up-regulated more than 8 fold in periodontitis tissue as compared to healthy gingivae. The expression of twenty-two miRNA genes (including let-7a, let-7b, let-7d, let-7e, let-7f, let-7i, miR-101, miR106b, miR-125a, miR-125b, miR-130b, miR-181b, miR-19b, miR-211, miR-23a, miR-23b, miR-30a, miR-30d, miR-340, miR-34c, miR-381, and miR-548d) were increased more than 4 fold (Fig. 2).

Validation of miRNA microarray results with quantitative real-time PCR

Real-time PCR was performed to confirm the differential expression of miRNA. All known miRNA are registered in a public web-based registry, the 'miRBase' database, which provides up-to-date information on all

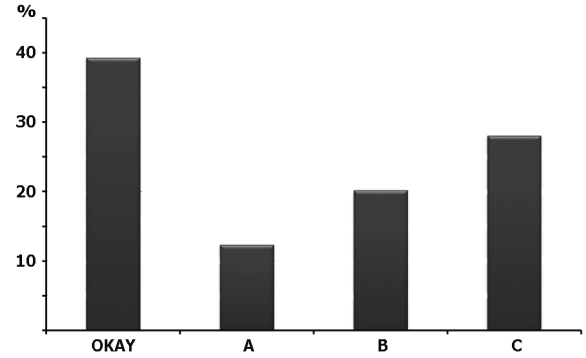

FIGURE 1. MiRNA microarray quality result. OKAY: The average threshold cycle of this gene is relatively high (> 30) in both the control and test sample. A: The average threshold cycle of this gene is relatively high (> 30) in either the control or test sample, and is reasonably low in the other sample (< 30). B: The average threshold cycle of this gene is relatively high (> 30), meaning that its relative expression level is low, in both the control and test samples, and the p-value for the fold-change is either unavailable or relatively high (p > 0.05). C: The average threshold cycle of this gene is either not determined or greater than the defined cut-off (default 35) in both samples, meaning that its expression was undetected, making this fold-change result erroneous and un-interpretable.

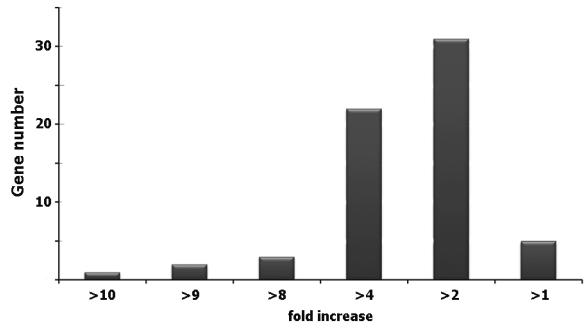

FIGURE 2. Number of genes increased or decreased in periodontitis gingiva as compared to healthy gingiva.

Table 1.

MiRNA microarray gene table

RT² miRNA PCR array Human Inflammation (MAH-105A)

let-7a	let-7b	let-7c	let-7d	let-7e	let-7f	let-7g	let-7i	miR-101	miR-106a	miR-106b	miR-125a-5p
miR-125b	miR-128	miR-130a	miR-130b	miR-1324	miR-144	miR-145	miR-15a	miR-15b	miR-16	miR-17	miR-181a
miR-181b	miR-181c	miR-181d	miR-186	miR-195	miR-19a	miR-19b	miR-202	miR-20a	miR-20b	miR-21	miR-211
miR-23a	miR-23b	miR-29a	miR-29b	miR-29c	miR-300	miR-301a	miR-301b	miR-302a	miR-302b	miR-302c	miR-30a
miR-30b	miR-30c	miR-30d	miR-30e	miR-340	miR-34a	miR-34c-5p	miR-372	miR-373	miR-374a	miR-374b	miR-381
miR-410	miR-424	miR-449a	miR-449b	miR-454	miR-497	miR-511	miR-513b	miR-519a	miR-519c-3p	miR-519d	miR-520d-3p
miR-520d-5p	miR-520e	miR-524-5p	miR-543	miR-545	miR-548a-3p	miR-548c-3p	miR-548d-3p	miR-548e	miR-590-5p	miR-607	miR-656
miR-875-3p	miR-9	miR-93	miR-98	SNORD48	SNORD47	SNORD44	RNU6-2	miRTC	miRTC	PPC	PPC

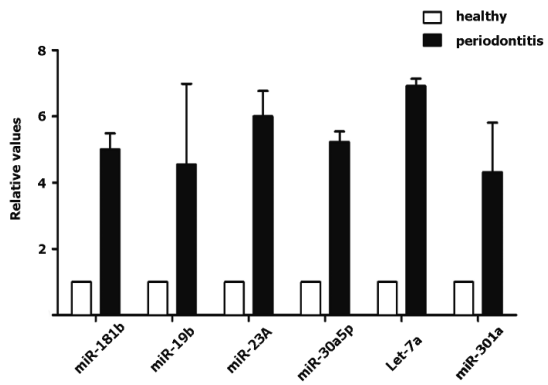

FIGURE 3. Real-time PCR results of the six miRNAs expressed differentially in periodontitis tissue compared to healthy tissue. The results were normalized using the RNU44 expression level and are presented as the mean ± standard error of the mean.

published miRNA. For real-time PCR to verify the microarray results, eight miRNA genes which were increased more than 4 fold in the microarray results and are known to be closely related to inflammation were selected (miR-181b, miR-19b, miR-23a, miR-30a, miR-let7a, and miR-301a, miR-520d-3p, and miR-548a-3p). Table 4 summarizes the target sequences of these eight miRNA genes. Six of these miRNA genes (miR-181b, miR-19b, miR-23a, miR-30a, miR-let7a, and miR-301a) were amplified successfully, as shown in figure 3, which suggests that these 6 miRNA genes may play a role in chronic periodontitis.

Discussion

In the present study, using miRNA microarray and real-time PCR, miR-181b, miR-19b, miR-23a, miR-30a, miR-let7a and miR-301a were up-regulated in the inflamed periodontal tissue of moderate-to-advanced chronic periodontitis patients. This study is the first report of periodontitis-related miRNA.

Table 2.

Microarray results for 93 human inflammatory miRNAs expressed differentially in healthy and periodontitis gingiva.

miRNA	fold	comment	miRNA	fold	comment	miRNA	fold	comment	miRNA	fold	comment
let-7a	9.48	OKAY	miR-17	2.37	OKAY	miR-302a	9.5	C	miR-511	4.32	B
let-7b	4.72	OKAY	miR-181a	1.21	A	miR-302b	9.5	C	miR-513b	9.5	C
let-7c	9.52	OKAY	miR-181b	4.64	OKAY	miR-302c	9.5	C	miR-519a	9.5	C
let-7d	4.68	OKAY	miR-181c	2.37	B	miR-30a	4.76	OKAY	miR-519c-3p	9.5	C
let-7e	4.74	OKAY	miR-181d	2.18	B	miR-30b	2.4	OKAY	miR-519d	9.5	C
let-7f	4.73	B	miR-186	1.19	A	miR-30c	2.38	OKAY	miR-520d-3p	8.77	B
let-7g	2.37	OKAY	miR-195	2.38	OKAY	miR-30d	4.75	OKAY	miR-520d-5p	9.5	C
let-7i	4.74	OKAY	miR-19a	2.38	OKAY	miR-30e	2.4	A	miR-520e	9.5	C
miR-101	4.74	A	miR-19b	4.79	OKAY	miR-340	4.37	B	miR-524-5p	9.5	C
miR-106a	2.36	OKAY	miR-202	2.36	A	miR-34a	2.39	A	miR-543	9.5	C
miR-106b	4.7	OKAY	miR-20a	2.39	OKAY	miR-34c-5p	4.28	B	miR-545	9.5	C
miR-125a-5p	4.71	OKAY	miR-20b	2.39	OKAY	miR-372	9.5	C	miR-548a-3p	8.65	B
miR-125b	4.78	OKAY	miR-21	2.35	OKAY	miR-373	9.5	C	miR-548c-3p	9.5	C
miR-128	2.39	OKAY	miR-211	4.43	B	miR-374a	2.36	OKAY	miR-548d-3p	4.32	B
miR-130a	18.83	B	miR-23a	4.76	OKAY	miR-374b	2.38	OKAY	miR-548e	9.5	C
miR-130b	4.34	B	miR-23b	4.78	OKAY	miR-381	4.37	B	miR-590-5p	2.38	B
miR-1324	9.5	C	miR-29a	2.37	OKAY	miR-410	9.5	C	miR-607	9.5	C
miR-144	9.5	C	miR-29b	2.37	B	miR-424	1.19	OKAY	miR-656	9.5	C
miR-145	1.2	A	miR-29c	2.35	OKAY	miR-449a	2.39	B	miR-875-3p	9.5	C
miR-15a	2.38	A	miR-300	9.5	C	miR-449b	9.5	C	miR-9	2.37	A
miR-15b	2.38	OKAY	miR-301a	8.59	B	miR-454	2.39	OKAY	miR-93	2.38	OKAY
miR-16	2.38	OKAY	miR-301b	9.5	C	miR-497	2.37	A	miR-98	2.37	A
ii	ii	ii	ii	ii	ii	ii	ii	ii	SNORD48	1.19	B

Recent evidence has shown that miRNA plays a key regulatory role in the immune response to pathogens and stimulus (O'Connell *et al*., 2007; Tili *et al*., 2007; Perry *et al*., 2008). However, the relationship between a bacterial infection and miRNA is just beginning to be explored with only a few reports published. Xiao *et al.* (2009) demonstrated the increased expression of miR-155 and miR-146a in human gastric epithelial cells infected with *Helicobacter pylori*, as well as the miRNA that play a potential role in a negative feedback loop to modulate the inflammation during a *H. pylori* infection (Xiao *et al*., 2009).

This study confirmed that 6 miRNA genes (including miR-181b, miR-19b, miR-23a, miR-30a, miR-let7a,

Table 3.

Microarray results including miRNAs analyzed only as OKAY, A, and B.

miRNA	fold	miRNA	fold	miRNA	fold	miRNA	fold
let-7a	9.48	miR-145	1.2	miR-21	2.35	miR-374a	2.36
let-7b	4.72	miR-15a	2.38	miR-211	4.43	miR-374b	2.38
let-7c	9.52	miR-15b	2.38	miR-23a	4.76	miR-381	4.37
let-7d	4.68	miR-16	2.38	miR-23b	4.78	miR-424	1.19
let-7e	4.74	miR-17	2.37	miR-29a	2.37	miR-449a	2.39
let-7f	4.73	miR-181a	1.21	miR-29b	2.37	miR-454	2.39
let-7g	2.37	miR-181b	4.64	miR-29c	2.35	miR-497	2.37
let-7i	4.74	miR-181c	2.37	miR-301a	8.59	miR-511	4.32
miR-101	4.74	miR-181d	2.18	miR-30a	4.76	miR-520d-3p	8.77
miR-106a	2.36	miR-186	1.19	miR-30b	2.4	miR-548a-3p	8.65
miR-106b	4.7	miR-195	2.38	miR-30c	2.38	miR-548d-3p	4.32
miR-125a-5p	4.71	miR-19a	2.38	miR-30d	4.75	miR-590-5p	2.38
miR-125b	4.78	miR-19b	4.79	miR-30e	2.4	miR-9	2.37
miR-128	2.39	miR-202	2.36	miR-340	4.37	miR-93	2.38
miR-130a	18.83	miR-20a	2.39	miR-34a	2.39	miR-98	2.37
miR-130b	4.34	miR-20b	2.39	miR-34c-5p	4.28	SNORD48	1.19

Table 4.

The target sequences of the eight miRNAs selected for real-time PCR.

miRNA-ID	sequence
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU
hsa-miR-181b	AACAUUCAUUGCUGUCGGUGGGU
hsa-miR-19b	UGUGCAAAUCCAUGCAAAACUGA
hsa-miR-23a	AUCACAUUGCCAGGGAUUUCC
hsa-miR-301a	CAGUGCAAUAGUAUUGUCAAAGC
hsa-miR-30a	UGUAAACAUCCUCGACUGGAAG
hsa-miR-520d-3p	AAAGUGCUUCUCUUUGGUGGGU
hsa-miR-548a-3p	CAAAACUGGCAAUUACUUUUGC

and miR-301a), were up-regulated in periodontitis tissue through real-time PCR after miRNA microarray profiling. A dysregulation of miR-181b is obviously connected to the development of drug resistance. There is a report showing that miR-181b is strongly associated with the response to 5-fluorouracil-based antimetabolite S-1 in various types of cancer (Hummel *et al*., 2010). In glioblastoma patients, miR-181b and miR-181c were down-regulated in response to chemoradiotherapy in comparison to advanced cases (Slaby *et al*., 2010). MiR-19b suppresses breast cancer cell proliferation and tumor colony formation as a tumor suppressor by acting as a miR-17-92 cluster (Zhang *et al*., 2009). The overexpression of miR-23a together with 27a and 24-2 in HEK293T cells induces apoptosis via a caspase-dependent and a caspase-independent pathway, indicating possible roles as an anticancer target (Chhabra *et al*., 2009). More recently, it was reported that the miR-23a cluster inhibits B cell development (Kong *et al*., 2010). MiR-30a was found to be significantly down-regulated in lung squamous cell carcinoma as compared to normal lung tissues (Yang *et al*., 2010) and biliary miR-30a is essential for biliary development in zebrafish, demonstrating the functional role of miR-30a in hepatic organogenesis (Hand *et al*., 2009). Let-7 miRNAs are a family of miRNAs strongly expressed in somatic cells and stabilizes the self-renewing versus differentiated cell fates (Melton *et al*., 2010). In this study, the expression of all 8 members of the let-7 family (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i let-7a and let-7c miRNAs), which were included in microarray, were higher in the periodontal tissue as compared to healthy control tissue. In particular, the let-7a and let-7c miRNA genes were increased more than 9 fold as compared to normal tissue in microarray analysis. Recently, it was reported that multiple members of the highly conserved let-7 miRNA family were the most abundant miRNAs in the lung, and the inhibition of let-7 miRNAs *in vivo* profoundly inhibited the production of allergic cytokines, such as IL-13, demonstrating the proinflammatory role of let-7 miRNAs in allergic lung disease (Polikepahad *et al*., 2010). Finally, miR-301a is the most potent NF-kB activator. MiR-301a activates NF-kB by negatively regulating the expression of the NF-kB-repressing factor (NKRF) gene (Lu *et al*., 2010). Moreover, the expression of miR-301a is up-regulated when NF-kB is activated. Therefore, a positive feedback loop exists for persistent NF-kB activation, whereby miR-301a down-regulates the NKRF levels leading to elevated NF-kB activity, which in turn further promotes miR-301a transcription (Lu *et al*., 2010). Periodontopathic bacteria or their LPS were reported to activate NF-kB, leading to the production of proinflammatory cytokine, such as TNF-α or IL-1β, which are related to local tissue destruction in periodontitis (Jotwani *et al*., 2010; Diya *et al*., 2008). Therefore, it is proposed that inflammatory stimuli including subgingival bacteria or their components, such as LPS, up-regulate miR-301a expression, which in turn activates NF-kB to make local cells to produce proinflammatory cytokines, leading to inflammation in periodontal tissue. Further study will be needed to test this hypothesis.

Until now, the increased miRNA levels in periodontitis tissue were examined. Most of them, except for let-7 miRNA and miR-301a, have been investigated in relation to cancer. In particular, there is no report showing that the miRNAs mentioned above are related to periodontitis. Therefore, each miRNA can be a potential target for periodontitis in a future study. Nevertheless, further study of more miRNAs is needed. Individual miRNA has the ability to modulate multiple genes, and many genes can be regulated by more than one miRNA. Therefore, the increased function of miRNA in periodontitis tissue may be more complex. Nevertheless, this study may provide insight into the unique miRNA response in periodontitis. Furthermore, these periodontitis-related miRNAs could become a new diagnostic marker and therapeutic target for the modulation of periodontitis.

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