MicroRNA expression profiling of nicotine-treated human periodontal ligament cells

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Abstract: Cigarette smoking is a lifestyle-related risk factor involved in the causation and progression of periodontal disease. Nicotine is a key toxic component of tobacco. However, the mechanisms underlying nicotine-induced periodontitis have not yet been fully elucidated. The present study investigated the microRNA (miRNA) expression profile of human periodontal ligament cells (PDLCs) treated with nicotine. Using differential analysis of miRNA array data, several differentially expressed miRNAs were identified in nicotine-treated PDLCs. Quantitative real-time PCR was employed to verify the accuracy of the miRNA array, and the targets of these dysregulated miRNAs were further analyzed. Function and pathway enrichment of differentially expressed miRNAs suggested that several important signaling pathways, such as the Toll-like receptor signaling pathway, nicotine addiction, the transforming growth factor-beta signaling pathway, and the hypoxia inducible factor-1 signaling pathway, are potentially responsible for nicotine-induced periodontitis. This study has helped to clarify the epigenetic mechanisms of nicotine-induced periodontitis, highlighting novel biomarkers and therapeutic targets.

Keywords: microRNA expression profiling; human periodontal ligament cell; nicotine; smoking.

Introduction

Periodontitis is the most common oral disease, often affecting the middle-aged and elderly populations and steadily damaging both the soft tissue and bone tissue that support the teeth (1,2). Periodontitis causes chronic inflammation around the teeth, leading to remodeling and destruction of the periodontium (3). Therefore, the goal of periodontitis therapy is not only to halt the progression of periodontal disease but also to encourage regeneration of the damaged periodontium (4). Periodontal ligament cell (PDLC), a type of multipotent stem cell, reside in the perivascular space between the alveolar pocket and the periodontal fibers (5,6). PDLCs are primarily responsible for the regeneration of periodontal tissues, as demonstrated in recent studies (7). Under various circumstances, including mild inflammation, the proliferation and differentiation of PDLCs may be inhibited (8). Nicotine, the main toxic component of tobacco, is known to be highly addictive (9). Therefore, clarification of the molecular mechanism underlying nicotine-induced periodontitis is important for understanding the causal relationship between the two and for devising the most effective approach for periodontitis therapy.

MicroRNAs (miRNAs) are single-stranded RNAs
(ssRNAs, 21-23 nucleotides) that function as important regulators of most aspects of biology (10). Previous studies have investigated the differential expression of miRNAs in gingival biopsy samples obtained from both healthy individuals and patients with periodontal disease (11). Expression of 9 of 11 identified miRNAs (miR-30e, miR-15a, miR-106b, miR-185, miR-130a, miR-30d, miR-22, miR-103, miR-18a, miR-142-3p, and miR-210) was significantly increased in a background of periodontal disease and obesity (11). Target prediction identified a group of different mRNAs that are involved in several biological processes, including cytokines, chemokines, and specific collagen. These genes are involved in miRNA-related pathways (11). Another study has determined miRNA expression profiles in healthy and inflamed human gingival tissue. In total, 125 differentially expressed miRNAs were identified in inflamed gingival tissues compared with those in healthy gingival tissues, including 91 and 34 miRNAs with increased and decreased expression, respectively (12). Furthermore, previous studies have also demonstrated that eleven miRNAs were downregulated by >2-fold in diseased vs. healthy gingiva (13).

However, the expression patterns of miRNAs in nicotine-induced PDLCs remain unclear. In the present study, normal human periodontal ligament tissues from volunteers were collected for PDLC isolation, and PDLCs were cultured for nicotine treatment. miRNA array analysis was performed for nicotine-treated PDLCs. Six differentially expressed miRNAs were randomly selected for quantitative real-time PCR (qRT-PCR) assessment. Furthermore, the important miRNAs related to biological processes and signaling pathways were analyzed. The results obtained help to clarify the epigenetic mechanisms of nicotine-induced periodontitis and highlight novel biomarkers and possibly useful therapeutic targets.

Materials and Methods

Cell culture
Primary human PDLCs were isolated from explanted healthy periodontal ligament (PDL) as described previously (14). Growth medium containing 1% L-glutamine, Dulbecco’s modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum, 10,000 IU/mL penicillin G, 100,000 mg/mL streptomycin sulfate and 25 mg/mL amphotericin B was used to culture the cells with 5% CO2 at 37°C, in a 6-well plate. PDLCs at third passage were used for treatment with nicotine (Sigma, Cambridge, MA, USA).

MicroRNA array
A miRNA Isolation Kit (Qiagen, Duesseldorf, Germany) was used to isolate RNA from the PDLCs treated and untreated with nicotine. The RNA was used immediately or stored at −80°C. The quantity and purity of the RNA was measured by NanoDrop (ND-2000 spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA). The Affymetrix platform was employed to produce the miRNA expression profiles.

Differentially expressed miRNAs
A fold-change of >1.5 and a P-value of <0.05 (t-test) were used as criteria to select the differentially expressed miRNAs in three experimental samples. Hierarchical clustering using Euclidean distance was performed to examine the expression patterns of differentially expressed miRNAs.

qRT-PCR
A reverse transcription kit (Takara Bio, Inc., Otsu, Japan) and a real-time PCR kit (Takara Bio, Inc.) were employed for reverse transcription and PCR detection, respectively.

Gene ontology analysis and pathway analysis
Gene ontology (GO) analysis for biological processes (BP), molecular function (MF), and cellular components (CC) was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) software version 6.7. Fisher’s exact test was used to determine the significance of each GO-term. Functional similarity among GO terms was measured based on the value of fold enrichment. Redundancy Elimination and Visualization of Gene Ontology (REViGO) software was used to summarize and represent the statistically significant GO terms by excluding subsets that were redundant.

Pathway prediction analysis was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) online database and a P-value of <0.05 was set as a criterion for determining the significance of the predicted pathways.

Results
Nicotine inhibits PDLC proliferation
The Cell Counting Kit-8 (CCK8) assay was employed to determine the effect of nicotine on PDLC viability. Treatment with nicotine (2 mM) (15) for 12 h dramatically inhibited cell viability (Fig. 1A). Importantly, prolonging the nicotine treatment time from 24 to 48 h increased this inhibitory effect (Fig. 1A). The effect of nicotine concentration on PDLC viability was also assessed. As shown in Fig. 1B, treatment with 0.5 mM nicotine for 24
h significantly inhibited PDLC viability, and the inhibitory effect became stronger as the nicotine concentration increased. These findings demonstrated that the inhibitory effect of nicotine on PDLC viability was time- and concentration-dependent.

**miRNA expression profile**

To investigate the expression profile of miRNAs in the absence and presence of nicotine treatment, the GeneChip miRNA 4.0 Array, containing 5214 mature miRNAs, was employed. In the nicotine treatment group, 16 miRNAs were upregulated (hsa-miR-4730, 7977, 3928-3p, 493-5p, 126-3p, 6750-5p, 22-5p, 300-5p, 326, 6870-5p, 877-3p, and 589-3p) and 14 were down-regulated (hsa-miR-212-5p, 129-5p, 4750-5p, 550a-3-5p, 4783-3p, 766-3p, 3136-5p, 4430, 370-5p, 29c-3p, 6871-5p, 337-3p, 550b-2-5p, and 570-3p), as shown in Table 1 and Table 2, respectively. Furthermore, hierarchical clustering for the differentially expressed miRNAs was performed as shown in Fig. 2.

**Quantitative RT-PCR validation of miRNA expression**

To confirm the accuracy of the miRNA microarray data, qRT-PCR assays were performed to measure the expression of several randomly selected miRNAs, including miR-326, 330-5p, 877-3p, 129-5p, 570-3p, and 29c-3p. The qRT-PCR data confirmed that miR-326, miR-330-5p and miR-877-3p were highly and significantly expressed in the nicotine-treated PDLCs, whereas miR-129-5p, miR-570-3p and miR-29c-3p showed low expression in the presence of nicotine (Fig. 3). Collectively, the data for qRT-PCR were consistent with those for miRNA array, thus verifying the accuracy of the expression patterns of the differentially expressed miRNAs.

**Functional enrichment of predicted target genes**

To identify the targets of the miRNAs, the miRWalk database (16) was used to predict the potential targets for all up-regulated and down-regulated miRNAs (Tables 1, 2). For the above 30 identified miRNAs whose expression was altered by nicotine, 4862 genes were predicted to be their targets. To explore the functions of the differentially expressed miRNAs, GO analysis of biological processes (BP), molecular function (MF), and cellular components (CC) was performed using DAVID software. All of the dysregulated gene sets were included in the functional enrichment analysis. A κ similarity of >0.85 and FDR <0.01 were set as criteria for selecting significant GO terms. Figure 4 shows the top 10 statistically significant GO terms along with their enrichment score (Fig. 4 A,
C, E) and fold enrichment (Fig. 4 B, D, F). The functional relationship network structures of the statistically significant GO terms were summarized and represented by using REViGO software (Rudjer Boskovic Institute, Zagreb, Croatia), as shown in Fig. 5. Collectively, these data provided a bioinformatics basis for understanding the network of miRNAs showing nicotine-mediated alterations in expression.

Signaling pathway enrichment
The enriched signaling pathways of the predicted target genes of the differentially expressed microRNAs were then investigated using the KEGG database. Tables 3 and 4 list the signaling pathways for the up-regulated and down-regulated miRNAs, respectively. Among these pathways, the Toll-like receptor signaling pathway, apoptosis pathway, nicotine addiction pathway, hypoxia inducible factor-1 (HIF-1) signaling pathway and transforming growth factor-beta (TGF-beta) signaling pathway were included in the targets of up-regulated and down-regulated miRNAs.

Discussion
Smoking is one of the major causes of periodontitis (17), and nicotine is often responsible for bone loss and periodontal pockets (9). On the other hand, it has been acknowledged that miRNAs play very important roles in the regulation of gene expression and biological function. Therefore, it is important to characterize the miRNA expression patterns in nicotine-induced periodontitis and analyze their possible functions. In the present study, miRNA array was used to detect and select the miRNAs that were differentially expressed in nicotine-treated PDLCs, and qRT-PCR was performed to validate those expression patterns. Moreover, the potential biological functions and molecular signaling pathways affected by these miRNAs were analyzed. The aim was to explore the mechanisms and functions of miRNAs underlying nicotine-induced periodontitis and to provide clues to novel therapeutic targets.

In recent years, several studies have shown that miRNAs play critical roles in many fundamental and important biological processes. Prediction of miRNA-disease associations can contribute to understanding the pathogenesis of diseases, development of new drugs, and formulation of personalized diagnosis and treatment strategies. The Path-Based MiRNA-Disease Association (PBMDA) prediction model was proposed by integrating known human miRNA-disease associations, miRNA functional similarity, disease semantic similarity, and Gaussian interaction profile kernel similarity for miRNAs and diseases (18,19). Computational models have become important means for identification of novel miRNA-disease associations, and can select the most promising miRNA-disease associations for experimental validation. This approach significantly reduces the time and cost of biological experiments (20,21). In this study, biological processes (BP), molecular function (MF), cellular components (CC), and predicted pathways modulated by dysregulated miRNAs were identified using DAVID software and KEGG analysis. The results provided candidate biomarkers and therapeutic targets for nicotine-induced periodontitis, although further studies will be required to confirm these findings.

A previous study has reported that miR-133 and miR-590 are related to the atrial remodeling induced by
Fig. 4 Gene ontology analysis of dysregulated miRNAs targets. Enrichment score was calculated as a log scale of the 
P-value (Fisher exact/EASE score) for the members of a corresponding annotation cluster and the fold enrichment was calculated as (Count/Pop.Hits)/(List.Total/Pop.Total). Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) are presented according to their respective enrichment scores (A, C, E) and fold enrichments (B, D, F).

Fig. 5 Networks analysis of dysregulated miRNAs targets. Biological process (BP), cellular component (CC) and molecular function (MF) networks altered by the dysregulated miRNAs in the nicotine-treated group.
nicotine (22). miR-21 is also up-regulated by nicotine, promoting the epithelial-mesenchymal transition in esophageal cancer cells (23). In addition, miR-133 is down-regulated in cardiomyocyte apoptosis after nicotine treatment (24). However, no study has been conducted to determine specific miRNA effects and mechanisms in nicotine-induced periodontitis. As a high-throughput screening method, the miRNA microarray has been applied to screen differentially expressed microRNAs involved in occurrence and progression of diseases (25).

In a recent study, Solleti et al. demonstrated that smoking of electronic cigarettes affected the expression of 125 miRNAs (26). The present study systematically analyzed the expression patterns of miRNAs in nicotine-treated PDLCs. Among all of the mature miRNAs, 16 were up-regulated (hsa-miR-4730, 7977, 3928-3p, 493-5p, 1260b, 3128, 4286, 6511b-5p, 126-3p, 6750-5p, 22-5p, 330-5p, 326, 6870-5p, 877-3p, and 589-3p) and 14 were down-regulated (hsa-miR-212-5p, 129-5p, 4750-5p, 550a-3-5p, 4783-3p, 766-3p, 3136-5p, 4430, 370-5p, 29c-3p, 6871-5p, 337-3p, 550b-2-5p, and 570-3p). The expression profile of miRNAs in nicotine-treated PDLCs provided several potential targets that are involved in several important biological processes in PDLCs, such as growth and the immune response. Further studies will be needed to further validate and demonstrate the effect and mechanism of specific miRNAs in nicotine-induced periodontitis.

miRNA dysregulation by nicotine in PDLCs could be one of the epigenetic mechanisms underlying the addiction-related behavioral effects of nicotine (27). Nicotine may activate the metabolic pathways of PDLCs (28), thus altering the expression of miRNAs (27). Further studies are required to investigate the mechanisms underlying nicotine-induced alterations in the expression profiles of miRNAs.

The present analysis suggested that the Toll-like receptor signaling pathway might underlie nicotine-induced periodontitis. The Toll-like receptor signaling pathway can induce distinct patterns of gene expression responsible for innate immunity and acquired immunity (29). In addition, the HIF-1 signaling pathway, the TGF-beta signaling pathway, and the nicotine addiction signaling pathway that regulate the tissue microenvironment as well as the growth and immune responses of numerous cells were also found to be potentially involved in nicotine-induced periodontitis. These signaling pathways appear to exert complex but coordinated mechanisms underlying nicotine-induced periodontitis and would warrant further investigation.

In summary, the present data-driven analysis has revealed several dysregulated miRNAs and enriched pathways in nicotine-treated PDLCs. These data could shed light on miRNA-associated molecular mechanisms, candidate biomarkers and therapeutic targets for nicotine-induced periodontitis.

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References