FOXP3 DNA Methylation Levels as a Potential Biomarker in the Development of Periapical Lesions

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Abstract

Introduction: Epigenetic mechanisms, such as DNA methylation, can modify gene expression patterns without changing the DNA sequence, comprising a tool that cells use to lock genes in the "off" position. Variations in the methylation profile have been correlated to a variety of human diseases. Here, we hypothesize that DNA methylation in immune response–related genes may contribute to the development of periapical lesions. **Methods:** The DNA methylation patterns of 22 immune response–related gene promoters were evaluated in 137 human periapical granulomas, 8 apical cysts, and 31 healthy gingival tissues from 2 independent cohorts using a pathway-specific real-time polymerase chain reaction array (EpiTect Methyl II; Qiagen Inc, Valencia, CA). Messenger RNA expression analysis by qualitative polymerase chain reaction was also performed. SABiosciences's hierarchical clustering and methylation (Qiagen, Valencia, CA) and Prism6 software (GraphPad Software, Inc, La Jolla, CA) were used for data analysis. **Results:** *FOXP3* gene promoter showed the highest level of methylation in both periapical granulomas and apical cysts ($P < .001$), and methylation levels were inversely correlated with *FOXP3* messenger RNA expression in the lesions. Furthermore, *FOXP3* expression was prevalent in inactive lesions and was positively correlated with interleukin-10 and transforming growth factor beta levels. **Conclusions:** Our results suggest that *FOXP3* acts as a master switch governing the development and function of T-regulatory cells, whose functions include the inhibition of immune responses and temper inflammation. The observed differential methylation patterns of *FOXP3* in periapical lesions may be crucial in determining its suppressive activity and may be involved in periapical lesion development. *(J Endod 2015;41:212–218)*

Key Words

Apical periodontitis, DNA methylation, FOXP3

Apical periodontitis represents a local immune response to the progression of microorganisms from an infected root canal space to the periapical area resulting in bone resorption (1). Such a complex cascade of events includes the release of numerous proinflammatory mediators through the innate and adaptive immune response mechanisms, which, if uncontrolled, contributes to a persistent destructive inflammatory reaction (2). Intriguingly, these inflammatory mediators that trigger tissue destruction can also promote wound healing (3), suggesting that the nature, extent, and duration of the host response seem to play a major role in the determination of tissue healing versus destruction and a complex signaling network operates in the determination of lesion outcome (4–6). The healing process is thought to begin immediately after tissue injury, but multiple systemic and local factors might disturb the natural course of healing, resulting in a chronic, nonhealing lesion (7). Cytokines play a major role in inflammatory and immune responses within the bone microenvironment. The balance between pro- and anti-inflammatory mediators determines the outcome of resorption in bone destructive diseases, including periapical granulomas (8).

Aberrant changes in gene function are believed to be involved in a wide spectrum of human diseases (9). Recent evidence suggests that genetic predisposition of the host can contribute to an individual's susceptibility to persistent apical periodontitis (10–14). Polymorphisms in disease-relevant genes, such as cytokines and matrix metalloproteinases, have been shown to be associated with pulpal and periapical pathologies (10–12). Moreover, there has been some evidence pointing to the hypothesis that epigenetic factors may also play an important role in pulpal and periapical disease (15, 16).

Epigenetic modifications are important in the normal functioning of the cell, from regulating the dynamic expression of essential genes and associated proteins to repressing those that are un-needed (17). In contrast, epigenetic modifications could also result in alterations in functional gene networks that lead to inflammatory disease; thus, epigenetic markers could serve as important biomarkers in inflammatory diseases. DNA methylation, histone modifications, and non-coding RNA are 3 distinct epigenetic mechanisms. DNA methylation is considered the major epigenetic modification in mammalian genomes and is known to have profound effects on gene expression (17). It has been proposed that the expression and responsiveness of several inflammatory mediators are programmed through epigenetic mechanisms, such as DNA

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methylation (18–20). Recently, DNA methylation of the *IFNG* gene, which encodes interferon gamma, was suggested to contribute to differential gene transcription and protein levels of this cytokine in periapical granulomas and radicular cysts (16).

However, the potential impact of epigenetic mechanisms on the efficiency of wound healing mechanisms and in modulation of the host inflammatory immune response in a periapical lesion scenario remains unexplored. Here, we hypothesized that DNA methylation in immune response–related genes may contribute to the dynamics of periapical lesion development.

Materials and Methods

Subjects and Samples

This study was approved by the Committee for Protection of Human Subjects at the University of Texas Health Science Center at Houston, University of São Paulo, and Universidade Federal de Minas Gerais. Subjects were patients presenting with periapical lesions characterized radiographically as radiolucent lesions showing absence of the periodontal ligament space and discontinuity of the lamina dura. All samples were obtained from teeth without pulpal sensitivity and without endodontic treatment that had been referred for tooth extraction or endodontic apical surgery. Patients with medical conditions requiring the use of systemic modifiers of bone metabolism or other assisted drug therapy (ie, systemic antibiotics, anti-inflammatory, or hormonal therapy) during the last 6 months before initiation of the study, patients with pre-existing conditions such as periodontal disease, and pregnant or lactating women were excluded from the study.

Samples from 2 different subject cohorts (United States and Brazil) were included in this study. The United States samples were composed of 27 granulomas, 8 periapical cysts (patients aged 18–53 years), and 6 controls (healthy gingival tissue obtained from crown lengthening procedures or third molars extractions, patients aged 19–29 years). The Brazilian samples were composed of 115 periapical granulomas (patients aged 19–59 years) and 27 controls (patients aged 17–27 years).

Samples were collected and divided into 2 roughly similar fragments and stored in formalin and Tissue Tek solution (Sakura Finetek Inc, Torrance, CA) for further genomic DNA extraction. In the case of large lesions, whenever possible, samples were divided into 3 fragments and stored in formalin, Tissue Tek solution, and RNA Later (Ambion, Austin, TX) for genomic DNA and RNA extraction. Samples stored in formalin were submitted to routine histologic processing (formalin-fixed paraffin-embedded tissues) and sectioned for histopathologic analyses. Granulomas were defined by the presence of capillaries, inflammatory cells, fibroblasts, collagen, and macrophages and the absence of an epithelial lining. Periapical cysts comprised lesions in which cavities were further developed and lined by stratified squamous epithelium.

Isolation and Restriction Digestion of Tissue DNA

Genomic DNA was extracted from frozen tissue samples using the DNeasy Blood and Tissue Kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol. The tissue DNA extracts were digested in preparation for methylation analysis using the EpiTect Methyl DNA Restriction Kit (Qiagen Inc) according to the manufacturer's protocol. The method used by the EpiTect Methyl II PCR System is based on the detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme (21). These enzymes will digest unmethylated and methylated DNA, respectively. After digestion, the remaining DNA in each individual enzyme reaction is quantified by real-time polymerase chain reaction using primers that flank a promoter region of interest. The relative fractions of methylated and unmethylated DNA are subsequently determined by comparing the amount in each digest with a "mock" (no enzyme added) digest using a ΔCt method (21).

DNA Methylation Analysis

The digested DNA samples were used as templates for the analysis of the methylation profile of 22 cytokine genes (Table 1) using the EpiTect Methyl II Signature Human Cytokine Production PCR Array (SABiosciences, Qiagen, Valencia, CA). For the initial analysis, DNA sample pools of the United States (n = 35) and Brazilian (n = 115) periapical lesions were used as templates. For validation experiments, DNA from individual granuloma (n = 11) and cyst (n = 5) samples were used as templates. Reactions were performed in a 20-μL final volume in a ViiA7 Sequence Detection Instrument (Life Technologies, Carlsbad, CA). Experiments were performed in duplicate. Data output representing DNA methylation levels were determined and analyzed using the EpiTect Methyl DNA PCR Data Analysis software (Life Technologies, Carlsbad, CA).

Gene Expression Analyses

For the genes showing differential expression in the methylation array, 20 granulomas from the Brazilian cohort and 11 granulomas and 5 cysts from the United States cohort were used as templates to measure the expression of messenger RNA (mRNA) transcript levels using TaqMan chemistry (Invitrogen, Carlsbad, CA) in a quantitative real-time reverse-transcription polymerase chain reaction. Total RNA was extracted from tissue samples using TRIZOL reagent (Life Technologies, Grand Island, NY) following manufacturer's instructions. The integrity of RNA samples was checked by analyzing 1 μg total RNA on a 1.2% (w/v) denaturing formaldehyde-agarose gel. After RNA extraction, complementary DNA was synthesized by using 3 μg RNA through a reverse-transcription reaction using Super Script III Reverse Transcriptase (Invitrogen). Reactions were run in a 20-μL final volume using Taqman mRNA expression assays (Invitrogen) targeting forkhead box P3 (FOXP3), receptor nuclear factor kappa ligand (RANKL), osteoprotegerin (OPG), interleukin (IL)-10, and

TABLE 1. Composition of Epitect Methyl II Signature Human Cytokine Production PCR Array

Groups	Genes
T-cell function regulators	*BCL10, BCL3, FOXP3, HMOX1, IL12 A, MALT1, MAP3K7, SOD1, STAT5A, TRAF2, TRAF6*
B-cell function regulators	*BCL10, BCL3, INHA, INHBA, STAT5A*
Transcriptional regulators	*BCL10, BCL3, FOXP3, GATA3, IRF1, SMAD3, STAT5A*
Translational regulators	*BCL3, IGF2BP2*
Environment and intracellular stimuli responses	*BCL10, BCL3, ELA2, FOXP3, GATA3, HMOX1, IL12 A, INHA, INHBA, LTB, MALT1, MYD88, NOD1, SMAD3, SOD1, STAT5A, TLR2*
Cytokine production signaling molecules	*BCL10, BCL3, FOXP3, HMOX1, INHA, INHBA, LTB, MALT1, MAP3K7, MYD88, NOD1, SMAD3, SOD1, STAT5A, TLR2, TRAF2, TRAF6*