Methylation Pattern of the *IFN-γ* Gene in Human Dental Pulp

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Abstract

Introduction: DNA methylation is characterized by the addition of methyl groups in cytosines within cytosine-phosphate-guanine (CpG) islands. Unmethylated islands are related with transcriptionally active structure, whereas methylated DNA recruits methyl-binding proteins that promotes chromatin compaction. Although epigenetic events can influence the expression of cytokines, such events have not been investigated in dental pulp yet. The purpose of the present study was to evaluate the methylation status of the interferon γ (*IFN-γ*) gene in human dental pulp affected by inflammation compared with pulp tissue of impacted molar teeth and to verify the impact of methylation status in the expression pattern of the gene. **Methods:** Methylation-specific polymerase chain reaction (MSP) was used to verify the DNA methylation status of the *IFN-γ* gene in 16 human dental pulps affected by inflammation and in 16 pulp samples of impacted molar teeth. Histologic sections stained by hematoxylin-eosin were used for histopathological evaluation, and the expression of IFN-γ was assessed by quantitative real-time PCR (qPCR). **Results:** Although total methylation was observed in 43.75% of the samples of normal dental pulp tissues, partial methylation or unmethylation was found in 93.75% of the samples of inflamed pulp tissues. All the samples with total methylation in MSP showed no transcription of *IFN-γ*. The qPCR results showed expression of *IFN-γ* in 5 of 10 samples with partial methylation. **Conclusion:** The present study gives the first evidence of the possible participation of epigenetic events in the development of dental pulp inflammation. *(J Endod 2010;36:642–646)*

Key Words

Dental pulp, inflammation, interferon-gamma, methylation

Epigenetics is described as modifications in conformational structure of DNA that do not involve changes in the sequence, making it more or less accessible to transcription (1, 2). DNA methylation is the most common epigenetic event characterized by the addition of the methyl group in cytosines within cytosine-phosphate-guanine (CpG) regions (2). Unmethylated CpG islands are related with a transcriptionally active structure, whereas methylated DNA recruits methyl-binding proteins leading to chromatin compaction and preventing the binding of transcription factors (3, 4) (Fig. 1).

Evidence is emerging about the importance of epigenetic regulatory mechanisms in controlling the inflammatory response (5). Epigenetic changes occur in cytokine genes in human cells, affecting the ability of the cell to express cytokines (6). Despite the small number of studies that evaluated CpG methylation in cytokine genes, initial findings have offered insights into the importance of methylation in the pathogenesis of some inflammatory diseases, such as chronic gastritis (7), airway inflammation (1), periodontitis (8), and bronchial asthma (9).

Few studies evaluating the methylation pattern in the *IFN-γ* gene have been performed. CpG methylation in the interferon gamma (IFN-γ) promoter is considered a negative transcriptional regulator of IFN-γ production (10). IFN-γ is an important proinflammatory cytokine released mainly from T cells in response to antigenic stimulation and involved in activation of macrophages and endothelial cells (11). It also regulates the antigen-specific immune response and potentiates many of the actions of tumor necrosis factor α on endothelial cells (12). A high prevalence of IFN-γ messenger RNA in inflamed pulps from shallow caries has been detected, being the initial stage of pulpal inflammatory response characterized by a cell-mediated type 1 immune response (13). Because of the important role of IFN-γ cytokine in the development of pulp inflammation, the presence of methylation in the promoter region of this gene may interfere in the immune response elaborated during the inflammatory process.

Although epigenetic events can influence the expression of cytokines, it has not been investigated in dental pulp yet. The purpose of the present study was to evaluate the methylation status of the *IFN-γ* gene in human dental pulp affected by inflammation compared with pulp tissue of impacted molar teeth. In addition, we prompted to evaluate the association between *IFN-γ* gene methylation with the cytokine transcription. We found evidence for the first time that epigenetic events may be important to *IFN-γ* expression in human dental pulp.

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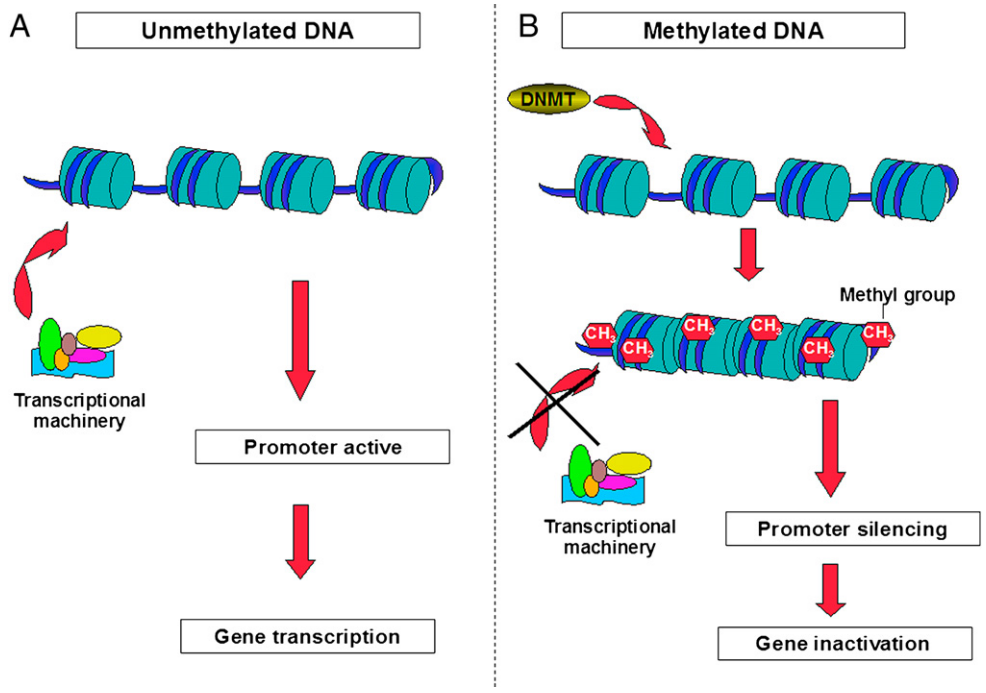

Figure 1. The effect of methylation in gene expression. (*A*) When the promoter region of DNA is unmethylated, there is an activation of gene expression. (*B*) DNA methylation occurs when methyl groups are added to cytosine nucleotides in specific areas of the gene by the enzyme DNA methyltransferase (DNMT). This chemical modification blocks the binding of transcription factors inhibiting gene transcription.

Materials and Methods

Sample Collection

A total of 32 samples of human dental pulp from individuals under treatment at the Dentistry School were included in this study. The samples were stratified into two groups: case group ($n = 16$) composed by pulp tissue from tooth with symptomatic pulpitis and the control group ($n = 16$) composed by pulp tissue from impacted third molars. The case group was composed of 8 female and 8 male subjects (age ranging from 11 to 57 years), and the samples were collected during conventional endodontic therapy. The control group was composed of 12 female and 4 male subjects (age ranging from 16 to 29 years), and the samples were collected after surgical removal of impacted third molars. Pulp tissues were immediately included in Tissue-tek (Sakura Finetek, CA) and stored at −80°C. For RNA extraction, a piece of the tissue was stored in RNAHolder (BioAgency Biotecnologia, Sao Paulo, Brazil) at −80°C. Because of the minute amount of tissue available, the extraction of DNA, RNA, and the analysis of microscopic sections were not possible to be done in all samples. The local ethics committee approved the present study.

Histologic Study

Two sections of 5 μm performed in 18 frozen samples were stained by hematoxylin-eosin for histopathological examination. The histological sections were submitted to inflammatory cell counting, and the mean number of inflammatory cells by a high-power field (original magnification × 400) was obtained.

DNA Extraction

The remaining fragments of the frozen samples were used to DNA extraction. The genomic DNA was extracted with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration of DNA was determined with a spectrophotometer.

Bisulfite Modification

Methylation pattern of tissues were assessed by using DNA modification by bisulfite treatment similar to that reported by Goldenberg et al (14). With the bisulfite treatment, unmethylated cytosines of DNA are converted to uracil, whereas methylated cytosines remain unmodified. The bisulfite conversion was performed with 1 μg of DNA, which was denatured by incubation with 2 μL of NaOH 3 mol/L for 20 minutes at 50°C. Then, the samples were treated with sodium bisulfite (2.5 mol/L) and hydroquinone (1 mol/L) for 3 hours at 70°C. Modified DNA samples were purified with Wizard DNA purification resin according to the manufacturer's instructions (Promega, Madison, WI) and eluted in distilled H_2O. NaOH (3 mol/L) was added to complete the modification, and this was followed by ethanol precipitation. The pellet was resuspended, and 200 ng of DNA were used for the polymerase chain reaction (PCR) assay.

Methylation-specific Polymerase Chain Reaction

Methylation-specific polymerase chain reaction (MSP) distinguishes the presence of methylation based on alterations produced after bisulfite treatment of DNA using specific primers for methylated or unmethylated DNA. The primers sequences used were previously described (15). All the samples were analyzed by MSP.

Polymerase chain reaction (PCR) was performed in a total volume of 25 $\mu\Lambda$ containing 2.5 μL 10× PCR buffer, 5 μL dNTP-mix (1 mmol/L of each), 0.75 μL of magnesium chloride (50 mmol/L), 0.5 μL of each primer (20 pmol/μL), 2.5 units of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA), and 200 ng of DNA. The amplification conditions consisted of 94°C for 4 minutes followed by 30 cycles of 94°C for 1 minute, 48°C for 1 minute and

72°C for 1 minute, and final elongation at 72°C for 7 minutes. The reactions were performed into a thermocyler (Eppendorf AG, Hamburg, Germany). PCR products were visualized in silver stained 6.5% polyacrylamide gel.

DNA treated with SssI methylase (New England Biolabs, Ipswich, MA) was used as positive control in methylated reactions, whereas DNA from peripheral blood mononuclear cells was used as control for unmethylated reactions, similarly to previous reports (16, 17). Positive amplification only for methylated primers was interpreted as total methylation. Positive amplification for both methylated and unmethylated primers were considered as partial methylation, according to the literature (18).

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted using Trizol reagent, and the RNA obtained was treated with RNAse-free DNAase I (Invitrogen Life Technologies) according to the manufacturer's protocol. The complementary DNA was synthesized from 1 μg of total RNA using superscript first-strand synthesis system (Invitrogen Life Technologies). Real-time polymerase chain reaction (qPCR) was performed using SYBR-green fluorescence quantification system in a Step-One real-time PCR 48-well plate (Applied Biosystems, Warrington, UK). The reaction conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 56°C for 1 minute. The sequences of primers used for IFN-γ were 5°-GGCATTTTGAAGAATTGGAAAG-3° (forward) and 5° -TTTGGATGCTCTGGTCATCTT-3° (reverse) as previously described (19). The primers used for b-actin were 5°- TGCCGACAGGATGCAGAAG-3° (forward) and 5°- CTCAGGAGGAGCAATGATCTTGA-3° (reverse) and were designed using PrimerExpress software (Applied Biosystems).

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems User Bulletin No. 2) as previously described (20), and the expression data were normalized with endogenous b-actin. The data were presented as the relative quantity of target IFN-γ normalized to endogenous b-actin and relative to a calibrator sample. As a calibrator, we used a pool of blood samples of healthy individuals.

Statistical Analysis

Statistical analysis of data was performed by using the Mann-Whitney test and Fisher exact test from BioEstat 4.0 software (Belém, Brazil). A p value of <0.05 was considered significant.

Results

MSP Results

MSP results of *IFN*-γ gene are illustrated in Figure 2. In the control group, 43.75% (n = 7) of the samples showed total methylation of *IFN*-γ gene (positive only for the methylated sequence of the gene), and 56.25% (n = 9) showed partial methylation (positive for both methylated and unmethylated sequences) (Fig. 2). The majority of the samples (93.75%) from case group showed positive unmethylated sequence. In the case group, 6.25% (n = 1) were totally methylated, 87.5% (n = 14) of the samples were partially methylated, and 6.25% (n = 1) were unmethylated (showed amplification only with the unmethylated primers) (Fig. 2). Representative data of the MSP reactions of *IFN*-γ gene are illustrated in Figure 3.

Histopathological Evaluation

A total of 18 samples was submitted to histopathological evaluation. The median number of inflammatory cells in the control group was 10, ranging from 2 to 75. In the case group, the median was 61, ranging from 23 to 180. The Mann-Whitney test showed increased number of inflammatory cells in the case group (p = 0.0025). The inflammatory infiltrate, when present, was predominantly mononuclear. Only two samples of the case group showed the predominance of polymorphonuclear neutrophils.

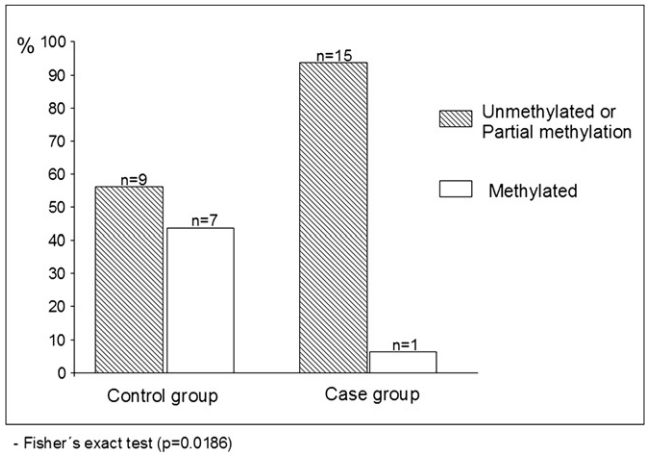

Figure 2. Results of the methylation-specific polymerase chain reaction (MSP) of the *IFN*-γ gene in dental pulp tissues.

Table 1 shows the MSP status from each case submitted to histopathological analysis. The mean number of inflammatory cells in the methylated samples was not statistically different from the unmethylated/partial methylated samples (p = 0.086).

qPCR Results

In order to evaluate the association between the methylation of *IFN*-γ and the transcription of the gene, the qPCR was performed in seven and six samples of the control and case groups, respectively (Table 2). Because undetectable levels of IFN-γ messenger RNA were observed in three of the six samples of the case group, no statistical difference was observed between IFN-γ expression in control versus case groups. However, the expression of the gene in the control group was clearly lower than that observed in the case group. All the three samples totally methylated did not show expression of IFN-γ. On the other hand, five of ten samples with partial methylation showed transcription of the gene ($2^{-\Delta\Delta Ct}$ ranging from 1.75 to 2457.09).

Discussion

The dental pulp may be considered a unique tissue because of its morphologic characteristics and its physical confinement (21). This tissue is protected from the oral environment by a rigid-walled pulp

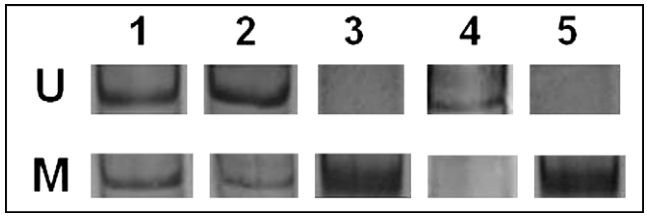

Figure 3. Representative data of MSP of the *IFN*-γ gene. U shows PCR products when amplified by unmethylated primers and M shows PCR products when amplified by methylated primers. Lane 1 and 2 shows samples with partial methylation. Lane 3 and 5 shows samples with total methylation. Lane 4 shows an unmethylated sample.

TABLE 1. Results of MSP of the *IFN-γ* Gene in Pulp Tissues from Impacted Third Molars (Control Group) and in Pulp Tissues from Teeth with Symptomatic Pulpitis (Case Group)

Samples	*IFN-γ* gene		Inflammatory cells	
Control group	Methylated	Unmethylated	Mean Number	Infiltrate
1	+	+	8	Mononuclear
2	+	+	5	Mononuclear
3	+	-	21	Mononuclear
4	+	+	2	Mononuclear
5	+	-	12	Mononuclear
6	+	-	3	Mononuclear
7	+	-	75	Mononuclear
8	+	+	20	Mononuclear
% of positivity	100%	50%		
Case group				
9	+	+	58	Mononuclear
10	+	+	23	Mononuclear
11	+	+	91	Mononuclear
12	+	+	64	Polymorphonuclear
13	+	+	123	Polymorphonuclear
14	+	+	23	Mononuclear
15	+	+	30	Mononuclear
16	-	+	37	Mononuclear
17	+	+	180	Mononuclear
18	+	+	115	Mononuclear
% of positivity	90%	100%		

Eight and six samples of the control and case groups, respectively, did not present sufficient tissue available for histological analysis.

chamber, which gives the dental pulp favorable conditions for investigation of methylation-inflammation interaction. The interaction between DNA methylation and inflammation may have relevance for inflammatory diseases, as the pulpitis. Microarray studies have shown different levels of 445 genes expression in the pulp of healthy and carious teeth (22). Furthermore, up-regulation of various cytokines messenger RNA were reported in symptomatic dental pulps (13, 23). Therefore, differentiated cytokines expression may occur during inflammatory reaction in dental pulp.

The pulp insult is a dynamic process that depends on both the invading microorganisms and host responses to them (24). Several cells and inflammatory mediators are involved in the initial pulpal responses to caries, including IFN-γ cytokine. IFN-γ activates macrophages to augment phagocytosis of microbes and promotes type 1 T-cell responses in adaptive immunity (25). IFN-γ has also been considered an important cytokine in the pathogenesis of experimentally induced pulp infection as endogenous suppressor of periapical lesions

TABLE 2. Results of MSP for *IFN-γ* gene and Expression of IFN-γ by Real-time PCR

Samples	*IFN-γ* gene		IFN-γ expression
	Methylated	Unmethylated	$2^{-\Delta\Delta Ct}$
Control group	+	+	Undetectable
	+	+	Undetectable
	+	-	Undetectable*
	+	+	1.75
	+	-	Undetectable*
	+	+	2.35
	+	+	Undetectable
Case group	+	+	2,457.09
	+	+	Undetectable
	+	-	Undetectable*
	+	+	15.5
	+	+	Undetectable
	+	+	142.22

*The three samples with total methylation did not show expression of IFN-γ.

development (26). Considering the important role of this cytokine in the inflammation of the dental pulp tissues, alterations in *IFN-γ* expression associated with the demethylation of the gene may influence the progression and development of the disease.

A few samples showed total methylation status, making clear association with density of inflammatory infiltrate difficult. Although total methylation of *IFN-γ* was observed in some samples of the control group, most of the samples in the case group exhibited partial methylation of this gene. This indicates that the presence of inflammation in human dental pulp is associated with loss of DNA methylation in the promoter region of the *IFN-γ* gene. Because unmethylated sequence of gene is correlated with high messenger RNA levels of IFN-γ in mice mononuclear cells (27), the loss of DNA methylation in the dental pulp may represent a defense mechanism mediated by IFN-γ production. To test the hypothesis of epigenetic regulation of IFN-γ production, we evaluated the transcription of this gene according to the MSP status. Although the totally methylated samples showed no transcription of *IFN-γ,* messenger RNA levels of *IFN-γ* were only observed in the samples with partial methylation.

Our study suggests that inflammation in human dental pulp is characterized by a change from a totally methylated to a partial methylated status of the *IFN-γ* gene. This finding may explain the high prevalence of IFN-γ messenger RNA in inflamed pulps and a cell-mediated type-1 immune response in the initial stage of pulp inflammation (13).

The demethylation of DNA can occur by blocking methylation of newly synthesized DNA during replication (28) or because of unknown enzymatic factor with demethylase activity (29) and also because of an oxidative demethylation (30). Although the process involving demethylation is poorly known, *IFN-γ* promoter demethylation was reported in memory CD8 T cells after antigenic stimulation (27).

In conclusion, our study shows that inflammation in human dental pulp is associated with demethylation of the promoter region of the *IFN-γ* gene. Furthermore, our study showed that the methylation status is associated with the transcription of the gene. These findings suggest that epigenetic events may be relevant to *IFN-γ* modulation in dental pulp. Further studies are necessary to determine the functional relevance of these alterations.

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