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TLR2 and *TLR4* gene promoter methylation status during chronic periodontitis

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Abstract

Aim: The objective of this study was to analyse the status of DNA methylation in the promoter region of the toll-like receptor (*TLR*)*2* and *TLR4* genes in gingival tissue samples from healthy subjects, smokers and non-smokers affected by chronic periodontitis.

Material and Methods: Genomic DNA and total RNA were purified from gingival tissue using the TRIZOL reagent protocol. Genomic DNA was then digested by methylation-sensitive restriction enzymes, amplified by polymerase chain reaction (PCR), electrophoresed on a 10% polyacrylamide gel and stained using SYBR Gold. Real-time PCR was also performed to verify the transcript levels.

Results: The CpG dinucleotides analysed were observed to be unmethylated in the majority of DNA samples of the three groups and statistical differences were not found among groups ($p > 0.05$). However, a trend towards methylation was observed in the *TLR2 Hha*I site in the samples of the periodontitis non-smoker groups. In fact, the analysis of all CpG sites together shows which complete methylation is observed in the shortest level in the samples of periodontitis non-smoker group.The analysis of transcript levels demonstrated no difference among groups ($p > 0.05$).

Conclusion: The results demonstrated major unmethylation of the *TLR4* gene promoter in all groups. However, the results for the *TLR2* gene promoter are inconclusive; this gene was found as a mosaic of methylated and unmethylated DNA in the majority of samples of the three groups and we also observed a trend towards the DNA methylation of CpG sites recognized by the *Hha*I enzyme.

Key words: CpG islands; epigenetic; inflammation; methylation; periodontitis; TLR; toll-like receptors

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Periodontal disease is triggered by infection, which leads to gingival inflammation and is followed by periodontal tissue destruction. However, these disease characteristics are the consequence of both bacterial presence and the host response, which is dependent on genetic and epigenetic components (Kornman 2008). Evidence indicates a key role for Gram-negative anaerobic microorganisms in the disease, including *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Tannerella forsythensis* (Pihlstrom et al. 2005). The innate-immune system recognizes conserved microbial products, termed pathogen-associated molecular patterns (PAMPs), which are invariant among diverse groups of microorganisms; PAMPs are recognized by a set of germline-encoded pattern recognition receptors, including toll-like receptors (TLRs) (Aderem & Ulevitch 2000, Hornef & Bogdan 2005).

Ten members of the TLR have been identified in mammals, and each TLR recognizes specific PAMPs. The prototypic Toll protein of *Drosophila*, a transmembrane receptor that was initially shown to control the dorsal–ventral patterning in the embryo, was also found

Conflict of interest and source of funding statement

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to be required for antifungal responses in adult flies (Gay & Keith 1991). Since then, 10 mammalian Toll homologues have been identified and all of these contain a leucine-rich ectodomain and a conserved cytoplasmic domain shared by both chains of the IL-1R, the IL-18R, SIGRR and MyD88 – the so-called Toll/IL-1R homologous region. Both genetic and biochemical data support a common signalling pathway that finally leads to the activation of NF-κB (Medzhitov & Janeway 2000a, b).

Cell wall components of periodontal pathogenic bacteria stimulate, via TLR2 and TLR4, the production of pro-inflammatory cytokines from the host, such as IL-1β, IL-8 and TNF, which induce alveolar bone resorption and the production of matrix metalloproteinases (MMPs) (Medzhitov & Janeway 2000a, b, Graves & Cochran 2003). Studies have shown that TLR2 mainly recognizes peptidoglycans and lipopeptides (Akira & Takeda 2004) and that TLR4 recognizes lipopolysaccharide (LPS) (Yoshimura et al. 2002, Darveau et al. 2004, Mochizuki et al. 2004). Furthermore, TLR2 can participate in the recognition of LPS and fimbrae of *P. gingivalis* (Asai et al. 2001, Hirschfeld et al. 2001). TLR4 participates in the recognition of LPS derived from *A. actinomycetemcomitans* and *F. nucleatum* (Yoshimura et al. 2002, Darveau et al. 2004, Mochizuki et al. 2004). Both TLR2 and TLR4 are constitutively expressed by epithelial cells and fibroblasts of oral tissues (Uehara et al. 2002, Kusumoto et al. 2004). In addition, some studies have shown that the expression of both receptors can be induced in oral epithelial cells (Uehara et al. 2002) and non-oral epithelial cells (Kikkert et al. 2007). Authors have also reported that *TLR2* and *TLR4* are more expressed in the gingival tissue of subjects with chronic periodontitis than in the gingival tissue of subjects with only gingivitis (Kajita et al. 2007). These data were confirmed by another study that observed higher numbers of cells expressing TLR2 and TLR4 in the periodontitis tissue than in the healthy tissue (Beklen et al. 2008). Upon ligand binding, TLR-mediated signalling activates signal transduction, leading to the transcription of cytokines and chemokines that initiate innate-immune responses (Akira et al. 2001).

Alterations in the methylation pattern have been described in oral diseases, such as squamous cell carcinoma (Kato et al. 2006), tongue carcinoma (Sinha et al. 2009) and odontogenic keratocyst (Moreira et al. 2009). Recently, we reported that the methylation pattern of the *IL8* gene promoter in individuals with chronic periodontitis is altered when compared with healthy subjects (Oliveira et al. 2009). In this study, we focused on the *TLR2* and *TLR4* genes. The methylation patterns of several CpG dinucleotides closely located to regulatory sequences in the promoter region of these genes were examined in order to investigate the relationship with chronic periodontitis.

Material and Methods

Subject population

This study was approved by the Institutional Review Board of the School of Dentistry of Piracicaba, University of Campinas. All patients were informed of the nature of the proposed treatment, and informed consent forms were signed. A convenience sample of unrelated subjects >25 years old, male and female, were recruited for the study. The patients were from the southeastern region of Brazil. All subjects were in good general health and had at least 20 teeth in the mouth. All subjects were subjected to anamnesis and to clinical and periodontal examination. Exclusion criteria included any systemic disorder that would affect the periodontal condition (with the exception of smoking), current pregnancy or lactation, and patients who had used systemic antibiotics or anti-inflammatory medication within 6 months before baseline. The smoking habit was recorded and patients who had consumed five cigarettes per day for at least 5 years were classified as smokers. Only patients who had never smoked were included in the non-smoker group. All subjects received a complete intra-oral and periodontal examination, including full-mouth periodontal probing, tooth mobility, gingival bleeding on probing, medical and dental anamnesis. The inclusion periodontal criteria were: (i) diagnosis and classification of generalized chronic periodontitis based on the 1999 Consensus Classification of Periodontal Diseases (Armitage 1999); (ii) good general health; (iii) diagnosis of chronic periodontitis characterized by the presence of periodontal pockets with clinical attachment loss (CAL) of $\geqslant 5$ mm and bleeding on probing; (iv) at least three teeth with a probing pocket depth of $\geqslant 5$ mm and bleeding after pocket probing in, at least, two different quadrants; and (v) $\geqslant 20$ teeth in both jaws. Measurements of probing pocket depth and CAL were recorded at six points around each tooth. The individuals of the control group were also examined with the same clinical parameter as that used in the chronic periodontitis group, but the subjects did not show any sites with CAL and probing pocket depth $\geqslant 3$ mm and presented full-mouth bleeding score (FMBS) $\leqslant 25\%$; tooth mobility was not found. None of the biopsied sites from the control subjects showed bleeding. Subjects were included in clinical categories according to periodontal health and smoking habit. Following these criteria, the groups were classified as follows:

- *Control healthy* (never smoked) ($n = 11$): Subjects exhibits no signs of periodontal disease, as determined by the absence of CAL and no sites with probing depth >3 mm.
- *Periodontitis smokers* ($n = 11$): Subjects with at least three teeth exhibiting sites ≥ 5 mm CAL in at least two different quadrants and had consumed five cigarettes per day for at least 5 years.
- *Periodontitis non-smokers* ($n = 12$): Subjects with at least three teeth exhibiting sites ≥ 5 mm CAL in at least two different quadrants.

Table 1 shows the clinical and demographic characteristics of the participants of the study.

Biopsy collection

Gingival biopsies were obtained from a single tooth of subjects undergoing periodontal surgery for periodontal disease-related (Tests Groups) and non-disease-related reasons (Control Healthy Group). Following block anaesthesia, tissues on the tooth surface where periodontal pockets were evaluated were incised to include the entire soft tissue walls of the pockets to be investigated. The entire pocket, junctional epithelia and connective tissue were removed.

Nucleic acid extraction

Samples of gingival tissue were obtained from biopsies and stored in a tube containing nucleic acid conserver (RNAholder, Bioagency, São Paulo, SP, Brazil). Samples were then frozen

Table 1. Mean values and standard deviation for the clinical parameters in the selected sites, and demographic and behavioural data in smokers and non-smokers

	Healthy ($n = 11$)	Smokers ($n = 11$)	Non-Smokers ($n = 12$)
Age (years)	39.8 ± 15.5	45.7 ± 7.4	45.5 ± 10.1
Gender % (male/female)	32.5/67.5	52.5/47.5	32.5/67.5
Probing depth (mm)	–	7.8 ± 1.9	7.4 ± 2.1
Full-mouth bleeding score (%)	⩽25	>25	>25
Cigarettes/day	–	16.5 ± 7.6	–
Cigarette consumption (years)	–	23.7 ± 9.1	–

at −70° C until analyses. Total DNA and RNA from gingival tissue cells was purified using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendation.

Methylation analysis

Polymerase chain reaction (PCR) analysis, relying on the inability of restriction enzymes to cut methylated sequences, was used to analyse the *TLR2* and *TLR4* gene promoters. The sites examined were recognized by one of the following restriction enzymes, whose activity was always blocked by CpG methylation: *Aci*I, *Hha*I, *Hpa*II and *Hpy*CH4IV.

Specific methylation-sensitive restriction enzymes

Genomic DNA (100 ng) was completely digested with the restriction enzymes in a total volume of microlitres, as recommended by the manufacturer (New Englands Biolabs, Beverly, MA, USA). The reaction of each restriction enzyme was conducted in an individual manner. The concentrations of the restriction enzymes, as well as the amount of DNA to be digested, have been described previously and calculated. Non-specific DNA digestion or DNA loss during the treatment, which will result in an underestimation of the level of methylation and incomplete digestion of non-methylated DNA, will result in an underestimation of the percentage of non-methylated DNA (Hashimoto et al. 2007).

PCR

A semi-quantitative PCR (qPCR) technique was performed in order to compare the methylation levels in the different groups. Therefore, a specific number of cycles of the PCR reaction for each gene studied were defined at the exponential phase of amplification. **TLR2**: After digestion, 10 μl of each solution (50 ng of DNA) was pipetted into a 25 μl PCR mixture containing 12.5 μl Go Taq Green Master Mix (Promega Corporations, Madison, WI, USA), 1 μl (10 pmol) each of primer: sense (5′-gaggtccagagttccctccg-3′) and antisense (5′-ccgggactaggaagtaagca-3′), with 127 pb. Primer sequences were derived from a *TLR2* gene promoter published by Haehnel et al. 2002 (GenBank accession number AC013303). The PCR was performed under the following conditions: 30 × (95°C, 1 min.; 63°C, 1 min.; 72°C, 1 min.). Subsequently, 10 μl of the PCR reaction was electrophoresed on a polyacrylamide gel stained with SYBR Gold (Invitrogen) and photographed. **TLR4**: After digestion, 5 μl of each solution (25 ng of DNA) was pipetted into a 25 μl PCR mixture containing 12.5 μl Go Taq Green Master Mix (Promega Corporations), 1 μl (10 pmol) of each primer: sense (5′-aaaggggaaatggggagtta-3′) and antisense (5′-ttttgcaggacacaatttgg-3′), with 393 pb (GenBank accession number AF172169). The PCR was performed under the following conditions: 30 × (95°C, 1 min.; 52°C, 1 min.; 72°C, 1 min.). Subsequently, 5 μl of the PCR reaction was electrophoresed on a polyacrylamide gel stained with SYBR Gold (Invitrogen) and photographed. Amplified DNAs of *TLR2* and *TLR4* genes were used as controls for restriction enzymes. An input of 100 ng of amplified DNA was submitted for the restriction of each enzyme to ensure their effectiveness. The non-enzyme-treated control DNA sample was always amplified with the primers, in parallel with the enzyme-treated samples, and both were subjected to electrophoresis in adjacent lanes. This provided a positive control for the PCR reaction and for DNA loading.

Quantification of methylation

In order to quantify the levels of methylation, electrophoretic bands were scanned and the absorbance was analysed using the Image J Software. Non-digested samples were considered to represent the total DNA template amplification (100% of the initial template copies were amplified). As such, the value of the quantification of enzyme-digested samples was subtracted from the value of the quantification of non-digested samples. This difference is the relative value of the non-amplified unmethylated DNA. Therefore, we classified the methylation levels into three groups: *unmethylated* = when the intensity of the band matched with 0–25% of the undigested DNA PCR band (0–25% of methylation); *partially methylated* = when the intensity of the band matched with 25–75% of the undigested DNA PCR band (25–75% of methylation); and *methylated* = when the intensity of the band matched with 75–100% of the undigested DNA PCR band (75–100% of methylation). DNA samples that were not treated with a restriction enzyme were considered as the control template, representing 100% of input DNA amplified after PCR. This analysis was performed for each restriction enzyme that recognized different CpG sites of the *TLR2* and *TLR4* gene promoters.

Expression analysis

Reverse transcription

One microgram of total RNA was treated with DNase (Invitrogen) and 500 ng was used for cDNA synthesis. The reaction was carried out using the SuperScript III First-strand Synthesis of the Oligo (dT) primer system (Invitrogen), following the manufacturer's recommendations. qPCR – a fragment of 243 bp, using specific primers for *TLR2* (forward: 5′-gccagcaggttcaggatgtc-3′/reverse: 5′-aggcatcccgctcactgtaa-3′), and a fragment of 210 bp, using specific primers for *TLR4* (forward: 5′-tcaaggaccagaggcagctc-3′/reverse: 5′-agccagcaagaagcatcagg-3′), were amplified by a qPCR that was performed in the LightCycler® system (Roche Diagnostics GmbH, Indianapolis, IN, USA) using the Fast-Start DNA Master Plus SYBR Green kit (Roche Diagnostic GmbH). The reaction product was quantified using the Relative Quantification tool (LIGHTCYCLER® Software 4; Roche Diagnostics GmbH), with glyceraldehyde-3-phosphate dehydrogenase as the reference gene.

Statistical analysis

The methylation frequency was compared for each individual site among

healthy and periodontitis groups using the χ^2-test at a significance level of 5%. With respect to gene expression analysis, an inter-group analysis was performed using the Kruskal–Wallis test at a level of 5%.

Results

In the present study, we used restriction enzymes whose performances are sensitive to methylated CpG as a methodology. Thus, the absence of PCR amplification indicates the absence of methylation and the presence of bands indicates partial or total DNA methylation. The CpG dinucleotides investigated in the *TLR2* and *TLR4* gene promoters are located close to important transcription sites. These regions respond to transcription factors such as NF-κB and Sp-1 and the region schemes are shown in Figs 1 and 2. We observed that the band intensities of enzyme-digested and non-enzyme-digested samples were clearly different, indicating that the methylation patterns in the CpG dinucleotides are not identical in the whole cells. The intensities of the bands in the acrylamide gel obtained after DNA digestion of some samples were visibly weaker and this was observed for both the *TLR2* and the *TLR4* genes.

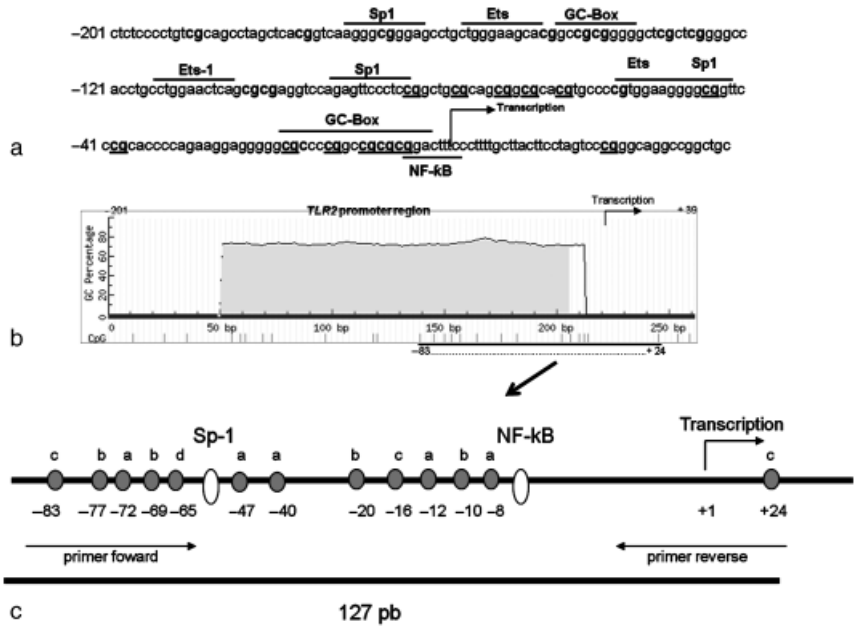

Fig. 1. The human toll-like receptor (*TLR*)*2* gene promoter. (a) Genomic sequence of the *TLR2* promoter region ranging from -201 to $+39$ pb is presented (GenBank accession number AC013303, Haehnel et al. 2002). Sites for potential transcriptional factor binding are over- and underlined. CpG potential methylation sites are in boldface and the CpG sites studied in this work are underlined. (b) A diagrammatic representation of the *TLR2* gene promoter from -201 to $+39$ bp is shown. The shaded areas indicate CpG islands in this promoter region. The horizontal bars indicate the CpG sites studied. (c) Schematic representation of the human *TLR2* promoter. Binding sites for different transcription factors are indicated as a white circle. Enzyme sites are indicated as grey circles (a, *Aci*I; b, *Hha*I; c, *Hpa*II; d, *Hpy*CH4IV).

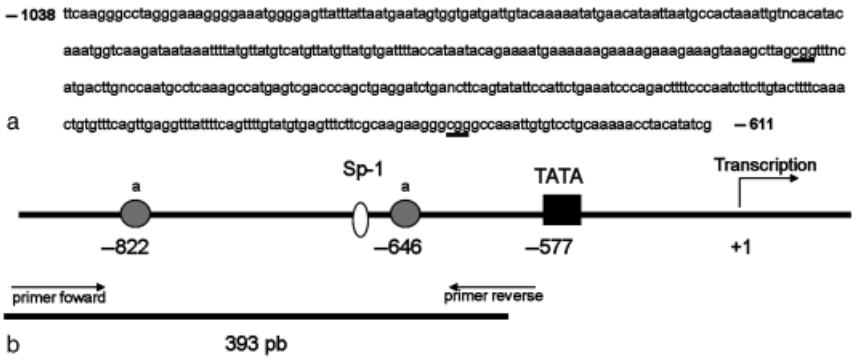

Fig. 2. The human toll-like receptor (*TLR*)*4* gene promoter.(a) Genomic sequence of the *TLR4* promoter region ranging from -1038 to -611 pb is presented (GenBank accession number AF172169). The CpG sites studied are underlined. (b) Schematic representation of the human *TLR4* promoter. Binding site for the transcription factor is indicated as a white circle. Enzyme sites are indicated as grey circles (a, *Aci*I).

TLR2

Most individuals presented a pattern of unmethylated sites, as the levels of methylation were between 0% and 25% in all the sites investigated. This pattern was observed for all groups and a statistical difference was not found between them ($p > 0.05$; χ^2). A minority of samples showed partially methylated sites (25–75%) or highly methylated sites (75–100%), as would be expected, as TLRs are key players in innate immunity and are constitutively expressed (Fig. 3). However, we observed a trend towards the methylation of the *Hha*I site in the periodontitis smoker and non-smoker groups, in contrast to the control group. When the results for the periodontitis smoker and non-smoker groups were analysed together and compared with the control group, a near significant difference was found ($p = 0.0848$; χ^2), and when the periodontitis non-smoker group was analysed compared with the control group, a significant difference was found ($p = 0.0278$; χ^2). Also, when the TLR2 results of all sites were combined, we found a statistical difference between groups ($p = 0.0119$; χ^2), and when the periodontitis non-smoker group was analysed compared with the control group, a stronger significant difference was found ($p = 0.0057$; χ^2), due to the few number of totally methylated samples in the periodontitis non-smoker group in contrast with other groups. The analysis of TLR2 transcript levels did not show any differences among groups ($p > 0.05$; Kruskal–Wallis) (Fig. 4).

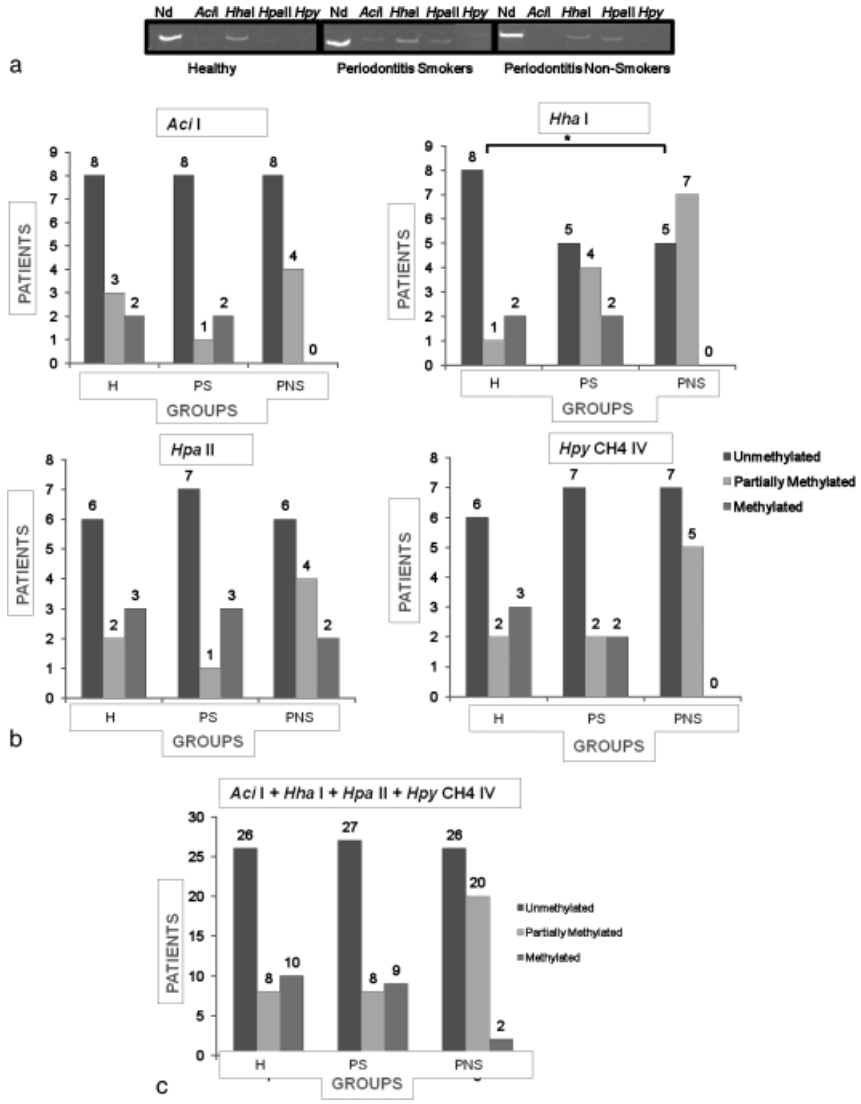

Fig. 3. Methylation analysis of the toll-like receptor (*TLR*)*2* gene promoter in gingival tissues. (a) Bands of representative samples of each group obtained after polymerase chain reaction of *TLR2* (127 pb); (Nd) indicates non-digested DNA samples, followed by digested DNA samples. Note that bands of digested DNA appear weaker than non-digested DNA. (b) Methylation frequency of the *Aci*I site ($p > 0.05$; χ^2); the *Hha*I site ($p < 0.05$; χ^2); the *Hpa*II site ($p > 0.05$; χ^2); and the *Hpy*CH4IV site ($p > 0.05$; χ^2). H, healthy ($n = 11$); PS, periodontitis smokers ($n = 11$); PNS, periodontitis non-smokers ($n = 12$); unmethylated, 0–25% of methylation; partially methylated, 25–75% of methylation; methylated, 75–100% of methylation in comparison with non-enzyme-digested DNA samples (100%). (c) Methylation frequency of *TLR2*, all sites' combination ($p = 0.0119$; χ^2).

TLR4

Similar to the *TLR2* gene promoter, the *TLR4* gene promoter was also unmethylated in most individuals, showing relative methylation levels of between 0% and 25% in the majority of samples. Total or partial DNA methylation was not observed among samples of the periodontitis non-smoker group and very few samples demonstrated these patterns in the control and periodontitis smoker groups. As such, no statistical differences were found among these groups ($p > 0.05$; χ^2) (Fig. 5). Transcript levels did not demonstrate differences among groups ($p > 0.05$; Kruskal–Wallis) (Fig. 6).

Discussion

Epigenetic modifications have been studied extensively in a variety of genes during carcinogenesis; however, the study of chronic inflammatory diseases has been neglected at this point. Hence, we investigated the methylation pattern of *TLR2* and *TLR4* genes in chronic periodontitis, as these proteins are implicated in the recognition of microorganism-derived molecules that trigger the periodontal inflammation process, and their expressions are up-regulated during gingival inflammation (Beklen et al. 2008).

The 5′-proximal region of the human *TLR2* gene lacks TATA boxes or consensus initiator sequences. Instead, it contains a CG-rich region that, according to the CpGPlot criteria (described by Takai & Jones 2002), characterizes this region as a typical CpG island (CGIs). Basal modulators of the transcription are present between nucleotides −281 and −41 (Haehnel et al. 2002) and several CpG methylation targets are found in this region. The CpG dinucleotides investigated in the *TLR2* gene promoter are located close to important transcriptions sites. Two *Aci*I sites (−47 and −40) are very close to the Sp-1 binding site; one *Aci*I (−8) and one *Hha*I (−10) are close to the NF-κB-binding site. It has already been shown that NF-κB has a central role in coordinating the expression of a wide variety of genes that control immune responses (Li & Verma 2002). NF-κB is one of the pivotal regulators of pro-inflammatory gene expression and induces the transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, MMPs, cyclooxygenase 2 and inducible nitric oxide synthase (Baeuerle & Baichwal 1997, Birbach et al. 2002). In addition, NF-κB and transcription factor Sp-1 represent the main factors responsible for the induction and constitutive transcription of the *TLR2* gene in murine (Wang et al. 2001) and human cells (Johnson & Tapping 2007). Recently, a specific region of the *TLR2* gene promoter, which contains two very close *Aci*I sites, was characterized as an important Sp-1-binding site in epithelial human cells. Additionally, this study showed that these sites were unmethylated in these cells, indicating a direct relationship between unmethylation and gene expression (Furuta et al. 2008).

The *TLR2* gene promoter methylation status was not different among healthy subjects and subjects with chronic periodontitis, independent of smoking habit. The CpG sites investigated were totally or partially unmethylated in the majority

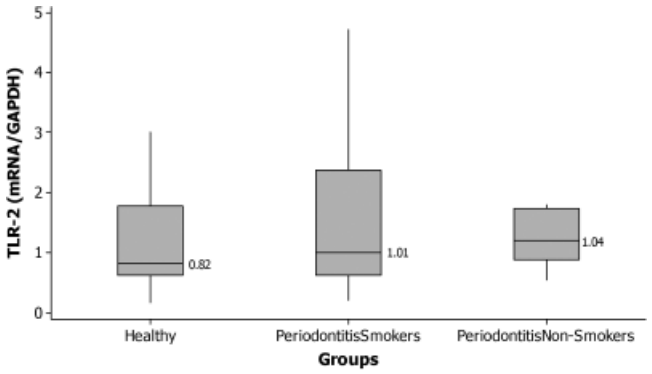

Fig. 4. Expression analysis of the toll-like receptor (*TLR*)*2* gene in gingival tissue. mRNA levels of healthy subjects and subjects with chronic periodontitis (healthy, $n = 11$; periodontitis smokers, $n = 11$; periodontitis non-smokers, $n = 12$) ($p > 0.05$; Kruskal–Wallis).

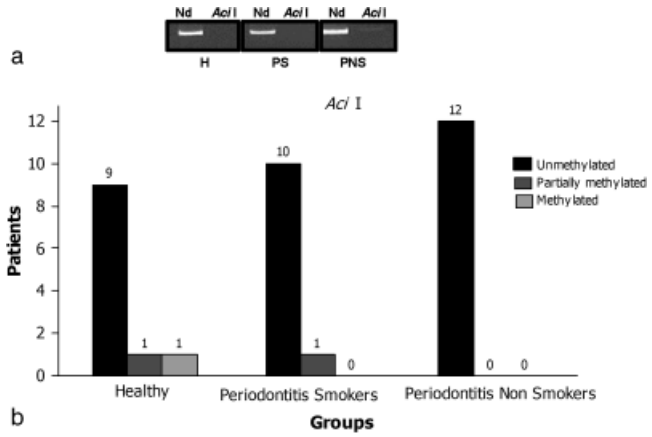

Fig. 5. Methylation analysis of the toll-like receptor (*TLR*)*4* gene promoter in gingival tissues. (a) Bands of representative samples of each group obtained after polymerase chain reaction of *TLR4* (393 pb); (Nd) indicates non-digested DNA samples, followed by digested DNA samples. (b) Methylation frequency of the *Aci*I site ($p > 0.05$; χ^2); H, healthy ($n = 11$); PS, periodontitis smokers ($n = 11$); PNS, periodontitis non-smokers ($n = 12$); unmethylated, 0–25% of methylation; partially methylated, 25–75% of methylation; methylated, 75–100% of methylation in comparison with enzyme non-digested DNA samples (100%).

of samples and this result is in accordance with another study that observed hypomethylation at these sites of the *TLR2* gene promoter in epithelial cells during cystic fibrosis (Shuto et al. 2006). Curiously, a trend towards the methylation status of *Hha*I sites was observed in the periodontitis samples and the results of the set of restriction enzymes confirmed this trend in the periodontitis samples. Whether this finding is a coincidence, due to the presence of four sites recognized by the *Hha*I restriction enzymes in the region, remains to be determined. However, importantly, *Hha*I represents the only enzyme able to recognize 5′ upstream CpG sites located close to the NF-κB and Sp-1 boxes.

The chromatin modification by DNA methylation is an important event, but is not the only mechanism of epigenetic regulation of gene expression. Hence, other epigenetic modifications may also regulate the transcriptional activity of the *TLR2* gene and must be investigated, particularly histone modifications such as H3K9, H3K27 and H3K4 methylation.

In contrast to the *TLR2* gene, the gene promoter of *TLR4* does not contain repeat sequences of CpG dinucleotides and does not represent a typical CGI promoter, as CpG sites close to the promoter are scarce. On the other hand, a TATA box located close to the Sp-1 sites is found in the *TLR4* promoter. TLR4 is up-regulated during gingival inflammation (Beklen et al. 2008); LPS binds to TLR4 on the cell surface and activates an intracellular signalling cascade through the TLR cytoplasmic Toll/IL-1 receptor-homology domain, culminating in the activation of NF-κB and AP-1 transcription factors. Therefore, TLR4 also participates in the activation of several genes involved in innate and adaptive responses, playing a key role in the LPS signal pathway (Poltorak et al. 1998, Arbour et al. 2000, Uehara et al. 2002).

Recently, a study reported that gene transcription of TLR4 is regulated by epigenetic mechanisms, including DNA methylation (Takahashi et al. 2009). However, we did not find any statistical differences in the transcript levels of this gene between the healthy group and the periodontitis groups. The methylation status of the *TLR4* gene promoter was also not found to vary among healthy subjects and subjects with chronic periodontitis, independent of smoking habit. However, we observed a consistent number of samples to be totally unmethylated in the *TLR4* gene, in contrast to the *TLR2* gene, whose results varied, and a number of samples were found to be partially unmethylated.

The role of tobacco as an epigenetic pattern modifier is not clear. Other studies have also reported inconclusive results regarding the contribution of smoking to the predisposition to epigenetic modification in lung cancer and periodontitis (Guo et al. 2004, Oliveira et al. 2009). On the other hand, increasing numbers of reports show a relationship between gene methylation, local infection and inflammation. Bacterial LPS was shown to induce de novo methylation in the CpG sites of mouse embryonic cells (Tatemichi et al. 2008) and epithelial cell lineages in gastric tumours (Katayama et al. 2009). In addition, *Helicobacter pylori* infection alters the DNA methylation status, increasing the risk of gastric cancer (Ushijima 2007) and HPV-positive tumours have also been associated with methylation pattern modifications (Richards et al. 2009).

Some reports have investigated whether inflammation is the cause or a consequence of gene methylation modifications. Authors have reported that chronic inflammation could predispose to the development of certain tumours through epigenetic alterations (Rutter et al. 2004, Hahn et al. 2008). During inflammation, cells of the innate immunity, mainly neutrophils and macrophages, induce the formation of reactive nitrogen species and reactive oxygen species that can damage proteins, lipids and DNA. In the latter case,

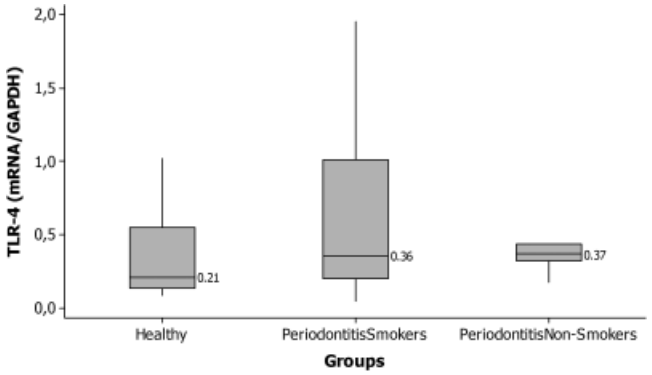

Fig. 6. Expression analysis of the toll-like receptor (*TLR*)*4* gene in gingival tissue. mRNA levels of healthy subjects and subjects with chronic periodontitis (healthy, $n = 11$; periodontitis smokers, $n = 11$; periodontitis non-smokers, $n = 12$) ($p > 0.05$; Kruskal–Wallis).

these alterations can occur as base mutations, deletions, sugar alterations, halogenation or oxidation. The oxidation of methyl groups changes the conformation of 5-methylcytosine, making it less recognizable to DNMT1, which, after mitosis, leads to loss of methylation. A second type of DNA methylation alteration is caused by reactive halogen compounds such as HOCl from activated neutrophils and HOBr from activated eosinophils. This results in methylcytosine mimicry, where DNA methyltransferase DNMT1 cannot distinguish between the halogencytosine and methylcytosine, leading to gain of methylation (Valinluck et al. 2006, Valinluck & Sowers 2007). Based on these observations, inflammation could lead to epigenetic modifications. In fact, some studies have shown that periodontal inflammation is associated with an alteration of promoter methylation in a variety of genes. We were one of the first groups to show this association. We already found aberrant methylation in the *IL8* gene promoter in chronic and aggressive periodontitis (Oliveira et al. 2009; Andia et al. 2010). Zhang et al (2010a) found a hypermethylation pattern of the *PTGS2* promoter in association with a lower level of *PTGS2* transcription and in another study they found a hypomethylation profile within the *IFN-γ* promoter region that was related to an increase of *IFN-γ* transcription (Zhang et al. 2010b). Viana et al. (2011) also verified the methylation status of the *IFN-γ* gene promoter, but they did not find aberrant methylation in the population studied. A recent study suggested that epithelial innate-immune responses are regulated by epigenetic modifications, and these responses are bacteria specific (Yin & Chung 2011). These authors showed that *F. nucleatum* induced *hBD2* and *CCL20* via both unmethylation and acetylation mechanisms, whereas the induction by oral pathogen *P. gingivalis* is only via the acetylation mechanism.

In the present study, a significant unmethylation was observed in the *TLR4* gene, whereas a trend towards methylation was observed in the *HhaI* CpG site in the *TLR2* gene. The promoter region of *TLR2* and *TLR4* contains many other CpG sites that were not studied in this work; thus, we cannot rule out the possibility that those CpG sites show an aberrant methylation status in inflamed tissues.

A previous study demonstrated that TLR1–9 are expressed by healthy gingival cells, as the oral cavity is not a sterile environment and TLRs represent sensors that are part of the innate-immune response against microorganisms. This same study still observed that TLRs expression is up-regulated during gingival inflammation (Beklen et al. 2008). Although the TLR2 and TLR4 mRNA levels have been increased during periodontitis, statistical differences in the TLRs transcription levels between healthy and periodontitis groups were not found in the present study. We cannot rule out bias in our samples as subclinical inflammation could have been present in the control samples and, also, individuals presenting a FMBS up to 25% were assumed to be healthy.

On the other hand, the decreased expression of the *TLR2* gene in the periodontitis group may be a reflection of the influence of increased levels of partial and total methylation in those samples. The DNA methylation status of CpGs present in the TLR genes must regulate mRNA transcription as the chromatin structure near the promoter region gene interferes with transcriptional activity (Bäckdahl et al. 2009). However, the genetic transcription represents a complex event that depends on several factors, such as: the epigenetic phenotype of interest gene and of all other genes that promote its regulation; transcription factors, cytokines and growth factors levels and their interactions and the time-point when the tissue biopsy was collected as quiescent phases occur during chronic periodontitis. Hence, the interference of methylation in the TLR genes cannot be discarded, but it is important to understand that CpG methylation represents one mechanism acting together with all other factors mentioned before.

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Clinical Relevance

Scientific rationale for the study: The orchestration of clinical phenotype during periodontitis is driven by the transcriptional regulation of genes that play an important role in this disease, where such alterations may affect disease prognosis. DNA methylation mechanisms are crucial for the regulation of gene expression, with consequent effects on cell phenotype. Several authors have reported an aberrant methylation status of genes associated with cancer and data demonstrate that DNA methylation is critical for regulating the inflammatory response in a dynamic manner. Therefore, the present study aimed to evaluate the DNA methylation status of the *TLR2* and *TLR4* gene promoters in samples of gingival tissue.

Principal findings: The methylation patterns of the *TLR2* and *TLR4* gene promoters were investigated in gingival tissues from healthy individuals, as well as in smoker and non-smoker individuals with periodontitis. The CpG dinucleotides analysed in the *TLR2* and *TLR4* gene promoters were observed to be unmethylated in the majority of DNA samples of the three groups and statistical differences were not found among groups.

Practical implications: The knowledge of the molecular mechanisms that control the genetic expression of proteins that participate in inflammatory diseases, such as periodontitis, may contribute to early disease diagnosis, helping to improve prognosis and provide new insights for the development of new and more effective therapies.