Lack of Association of a Functional Polymorphism in the Interleukin 8 Gene with Susceptibility to Periodontitis

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The important inflammatory mediator interleukin-8 (IL-8) is responsible for the migration and activation of neutrophils. The *IL8* gene contains a functional single-nucleotide polymorphism (SNP) (rs4073) in its promoter region that may influence the expression of IL-8, and which has been associated with inflammatory diseases. The purpose of this study was to investigate the association of the SNP (rs4073) in the *IL8* gene with susceptibility to periodontitis. DNA was extracted from the buccal epithelial cells of 500 individuals (control $n = 224$ and periodontitis $n = 276$). Individuals were genotyped for the SNP (rs4073) using sequence-specific primer polymerase chain reaction. Associations between the SNP (rs4073) and subject phenotypes were analyzed using the chi-squared test, followed by univariate and multivariate logistic regression modeling. The genotype distributions in both groups were consistent with Hardy–Weinberg equilibrium. Univariate and multivariate analysis showed that age, skin color, and smoking status were associated with periodontitis. No significant differences were found for sex and frequencies of alleles and genotypes between the control and periodontitis groups in the univariate analysis. These findings were replicated in the multivariate analysis. The SNP (rs4073) in the *IL8* gene is not associated with susceptibility to periodontitis in Brazilian individuals, even after controlling for covariates.

Introduction

PERIODONTAL DISEASES (PD), such as periodontitis and aggressive periodontitis, are characterized by a progressive gingival inflammatory response to the bacterial dental plaque, leading to destruction of periodontium and eventually tooth loss (Nibali *et al.*, 2007). PD are multifactorial diseases whose manifestation and progression are determined by the response of the host to bacterial infection (Kinane and Hart, 2003). The host response is influenced by both environmental factors (e.g., smoking, oral hygiene, stress, and lifestyle) and genetic factors (Albandar, 2002). Periodontitis has been associated with systemic alterations such as low-grade inflammation (Slade *et al.*, 2003), dyslipidemia (Katz *et al.*, 2002), glucose intolerance (Nibali *et al.*, 2007), endothelial dysfunction (Tonetti *et al.*, 2007), cardiovascular disease (Scannapieco *et al.*, 2003), and metabolic syndrome (D'Aiuto *et al.*, 2008).

Interleukin-8 (IL-8) is a potent chemoattractant and activator of neutrophils in inflammatory regions (Bickel, 1993). In the periodontal tissue, neutrophils are the first line of defense against periodontopathic bacteria, and play an important role in pathogenesis of periodontitis (Van Dyke and Vaikuntam, 1994). The role of IL-8 in the pathological process of chronic PD has been previously investigated. Levels of the IL-8 protein found in the gingival crevicular fluid from patients with chronic PD were significantly higher than those in controls. Expression of the *IL8* gene was detected in human periodontal ligament cells and gingival fibroblasts in culture after stimulation with lipopolysaccharides from periodontal pathogens, which could trigger infiltration of several inflammatory cells into the periodontal tissues (Tamura *et al.*, 1992; Yamamoto *et al.*, 2006). Consequently, strong inflammatory reactions can occur at local sites, resulting in the destruction of periodontal tissues (Okada and Murakami, 1998).

There has been a particular interest in investigating functional polymorphisms in candidate genes for association with disease. Functional single-nucleotide polymorphisms (SNPs) may cause phenotypic changes through multiple mechanisms. For example, they may change the sequence of a protein, or affect gene regulation, mRNA processing, and translation (Wang and Sadee, 2006). The SNP (rs4073) (reference sequence number from the NCBI's Entrez system) in the promoter region of the *IL8* gene is thought to be functional because its A allele has been shown to be associated with higher IL-8 production after stimulation with lipopolysaccharide (Hull *et al.*, 2000). It is worth mentioning that

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the SNP (rs4073) has previously been referred to by other identifiers, such as −251 (Hull *et al.*, 2000) and −353 (Renzoni *et al.*, 2000).

Several studies have demonstrated that SNPs in cytokine genes may influence the susceptibility to PD and their severity (Scarel-Caminaga *et al.*, 2002, 2004; Moreira *et al.*, 2007; Wagner *et al.*, 2007). However, to the best of our knowledge, the SNP (rs4073) in the *IL8* gene had never been studied in individuals with PD. Therefore, the aim of this study was to investigate whether or not this functional SNP in the *IL8* gene is associated with susceptibility to periodontitis in Brazilian individuals.

Materials and Methods

Selection of subjects

This study involved individuals from the State of São Paulo in the southeastern region of Brazil. A total of 500 subjects, all having similar socioeconomic status, were recruited from the patient pool of the School of Dentistry at Araraquara, UNESP—São Paulo State University, between November 2004 and May 2007. The study was approved by the Committee for Ethical Affairs of the São Paulo State University (Protocol number 57/04). All volunteers were informed about the aims and methods of this study, and gave their written consent to participate.

All subjects were required to have at least 10 remaining teeth and be in good general health. The exclusion criteria were a need for antibiotic prophylaxis, chronic usage of antiinflammatory drugs, current pregnancy, ongoing orthodontic therapy, and a self-declared history of systemic or local disease influencing the immune system, diabetes mellitus, HIV infection, or immunosuppressive chemotherapy.

Diagnosis of periodontitis was established through patient medical and dental histories. Each subject was examined by one of two calibrated periodontists, who carried out the periodontal examinations throughout the study period. To eliminate interexaminer variability, the kappa statistic test was calculated. Agreement of parameters between the dentists was tested for seven volunteers before the dental examinations (weighted kappa statistics = 0.74).

The following clinical signs and parameters were assessed at six sites around each tooth: probing pocket depth (PPD) and clinical attachment level (CAL, as the distance from the cement–enamel junction to the base of the periodontal pocket) measured to the nearest millimeter by a periodontal probe with Williams makings (Trinity-Campo, Mourão, Brazil), and bleeding on probing (BOP), registered as present or absent, as a percentage of the total number of sites. The subjects were categorized into two groups:

- Control group: subjects exhibiting no sites with CAL and PPD ≥ 3 mm and BOP.
- Periodontitis group: subjects exhibiting one or more sites with CAL and PPD ≥ 3 mm and BOP.

Information on smoking status was obtained using a self-reporting questionnaire. Smoking status was assessed and subjects were classified as a smoker or a nonsmoker, according to Kornman *et al.* (1997). Smokers were current smokers, and nonsmokers were subjects who had never smoked or who had not smoked within the last 5 years.

Analysis of genetic polymorphism

Buccal epithelial cells from the subjects were obtained using 3 mL of 3% glucose mouthwash for 2 min. DNA was extracted with sequential phenol/chloroform/isoamyl alcohol (25:24:1) solution and precipitated with salt ethanol solution. To genotype individuals for the SNP (rs4073) in the *IL8* gene, a sequence-specific primer polymerase chain reaction (SSP-PCR) was performed as previously described by Renzoni *et al.* (2000), using the following primers: *IL8* F-5′ GTGGAACTGATTTCTATGTGAA 3′, *IL8* R-5′ CACAATTT GGTGAATTATCAAT/A 3′, control *DRB* F-5′ TGCCAAGTG GAGCACCCAA 3′, and control *DRB* R-5′ GCATCTTGCTCT GTGCAGAT 3′. PCR amplification was confirmed using control primers for the *DRB* gene, which encodes a type of class II protein of from the major histocompatibility complex.

PCR was carried out using 1×buffer (pH 8.4, 20 mM Tris-HCl, and 50 mM KCl; Invitrogen, São Paulo, SP, Brazil), 0.2 mM of each dNTP (GE Healthcare Life Sciences, Buckinghamshire, England), 0.2 μM of each control primer (Invitrogen, Frederick, MD), 1.5 μM of each allele-specific primer (Invitrogen, Frederick, MD), 1.5 mM MgCl$_2$, 0.75 U Platinum *Taq* DNA polymerase (Invitrogen, São Paulo, SP, Brazil), and 100 ng of genomic DNA, with a final reaction volume of 13 μL. The cycling conditions were as follows: 1 min at 96°C; 5 cycles of 25 s at 96°C, 45 s at 70°C, and 25 s at 72°C; 21 cycles of 25 s at 96°C, 50 s at 65°C, and 30 s at 72°C; and 4 cycles of 30 s at 96°C, 60 s at 55°C, and 90 s at 72°C. The SSP-PCR products were analyzed by electrophoresis on a 10% polyacrylamide (USB, Cleveland, OH) gel, which was stained by the rapid silver staining method (Sanguinetti *et al.*, 1994).

Statistical analysis

The illustrative power calculations for periodontitis, for the estimation of the relevance of the *p*-values produced from this dataset, were performed using the methodology for discrete traits in case–control studies (Purcell *et al.*, 2003). The parameters considered were similar to those used by Brett *et al.* (2005), including a chronic periodontitis prevalence of 0.06 (Dini and Castellanos, 1995). Associations between the SNP (rs4073) and certain characteristics of the subjects (age, sex, skin color, and smoking status) in the control and periodontitis groups were analyzed using the chi-squared test, followed by univariate and multivariate logistic regression modeling. Statistical analyses were performed using the SAS statistical package v.9 (SAS Institute, Cary, NC). The BioEstat statistical package v.4.0 (UFPA, Belém, Brazil) was used to calculate Hardy–Weinberg expectations. Differences were considered significant when $p < 0.05$.

Results

The power calculations we performed showed that a sample size of 175 individuals would be required to ascertain a significant association between PD and the studied genetic polymorphisms, with an α-value of 0.001 and a power of 95%. Therefore, the number of subjects enrolled in this study is large enough to detect association with an acceptable level of confidence.

The characteristics of the Brazilian population investigated here are presented in Table 1. Subjects were categorized

TABLE 1. CHARACTERISTICS OF THE STUDIED GROUPS

	Control (n = 224)	Periodontitis (n = 276)	Total (n = 500)
Age (years), mean (±)	35.3 (±10.4)	43.4 (±10.5)	39.7 (±11.2)
Sex n (%)			
Female	134 (59.8%)	175 (63.4%)	309 (61.8%)
Male	90 (40.2%)	101 (36.6%)	191 (38.2%)
Skin color n (%)			
Whites	148 (66.0%)	142 (51.4%)	290 (58.0%)
Darker-skinned blacks	25 (11.2%)	62 (22.4%)	87 (17.4%)
Lighter-skinned blacks	45 (20.0%)	71 (25.7%)	116 (23.2%)
Yellow-skinned individuals	6 (2.8%)	1 (0.5%)	7 (1.4%)
Smoking habits n (%)			
Nonsmokers	204 (91.0%)	211 (76.4%)	415 (83.0%)
Smokers	20 (9.0%)	65 (23.6%)	85 (17.0%)

according to their skin color composition, as proposed by Peres *et al.* (2007), into whites (predominantly of European descent), darker-skinned blacks (predominantly of African descent), lighter-skinned blacks (mixed European, African, and Amerindian ancestry), and yellow-skinned individuals (Asian ancestry) (Table 1).

The SNP (rs4073) could be genotyped for 97.6% of the samples. Genotyping failed in four patients from the control group and eight patients from the periodontitis group (compare Tables 1 and 2), probably due to the high percentage of cytosines and guanines in this promoter region of the *IL8* gene. Nevertheless, the SSP-PCR method was efficient for genotyping (Fig. 1), as the genotypes of 10% of the samples (selected at random) were confirmed by the restriction fragment length polymorphism method (Rovin *et al.*, 2002). The observed genotype distribution was consistent with the assumption of the Hardy–Weinberg equilibrium in

both the control ($p = 0.106$) and periodontitis ($p = 0.136$) groups.

Table 2 shows the results of univariate analysis for association between the age, sex, skin color, smoking status, and genotypic distributions of patients and periodontitis. Significant associations between age (OR ranging from 3.62 for age group 30–39 to 27.1 for age group 60–69), skin color (OR = 1.69 for lighter-skinned blacks; OR = 2.66 for darker-skinned blacks), and smoking status (OR = 3.38) and periodontitis were observed; therefore, they were considered confounding factors. On the other hand, neither sex nor the SNP (rs4073) in the *IL8* gene was found to be associated with periodontitis.

To more accurately evaluate the strength of any association and to eliminate the distortion caused by confounding effects, multivariate analysis was performed. All the confounding factors detected by the univariate analysis were also observed by the multivariate analysis (Table 2). Even

TABLE 2. ODDS RATIOS FOR PERIODONTITIS BY CHARACTERISTICS OF THE PATIENTS (INCLUDING THE GENOTYPES OF THE SNP RS4073 IN THE *IL8* GENE)

| Characteristics of patients		Control	Periodontitis	Univariate OR (95% CI)	Multivariate OR (95% CI)	p-Value[a]
Total		220	268			
Age	20–29	82 (77.36)	24 (22.64)	Reference	Reference	<0.01
	30–39	66 (46.48)	76 (53.52)	3.628 (2.091–6.293)	3.392 (1.874–6.141)	
	40–49	53 (33.97)	103 (66.03)	6.262 (3.603–10.884)	6.601 (3.643–11.959)	
	50–59	15 (25.42)	44 (74.58)	9.364 (4.499–19.492)	10.488 (4.824–22.802)	
	60–69	2 (10.53)	17 (89.47)	27.134 (5.876–125.305)	41.215 (8.639–196.630)	
	>70	2 (33.33)	4 (66.67)	6.385 (1.105–36.874)	8.212 (1.354–49.803)	
Sex	Male	90 (47.12)	101 (52.88)	Reference	Reference	0.91
	Female	134 (43.36)	175 (56.64)	1.160 (0.804–1.672)	1.051 (0.687–1.608)	
Skin color	Whites	148 (51.03)	142 (48.97)	Reference	Reference	<0.01
	Darker-skinned blacks	25 (28.73)	62 (71.27)	2.665 (1.584–4.485)	2.820 (1.585–5.020)	
	Lighter-skinned blacks	45 (38.79)	71 (61.21)	1.696 (1.090–2.636)	1.824 (1.116–2.981)	
	Yellow-skinned individuals	6 (85.71)	1 (14.29)	0.179 (0.021–1.507)	0.362 (0.036–3.673)	
Smoking habits	Nonsmokers	204 (49.16)	211 (50.84)	Reference	Reference	<0.01
	Smokers	20 (23.53)	65 (76.47)	3.387 (1.960–5.854)	4.010 (2.205–7.292)	
SNP rs4073	AA	36 (39.13)	56 (60.87)	1.508 (0.877–2.593)	1.463 (0.789–2.712)	0.48
	AT	120 (45.11)	146 (54.89)	1.180 (0.775–1.796)	1.191 (0.732–1.939)	
	TT	64 (49.23)	66 (50.77)	Reference	Reference	

[a]Obtained from chi-squared test.
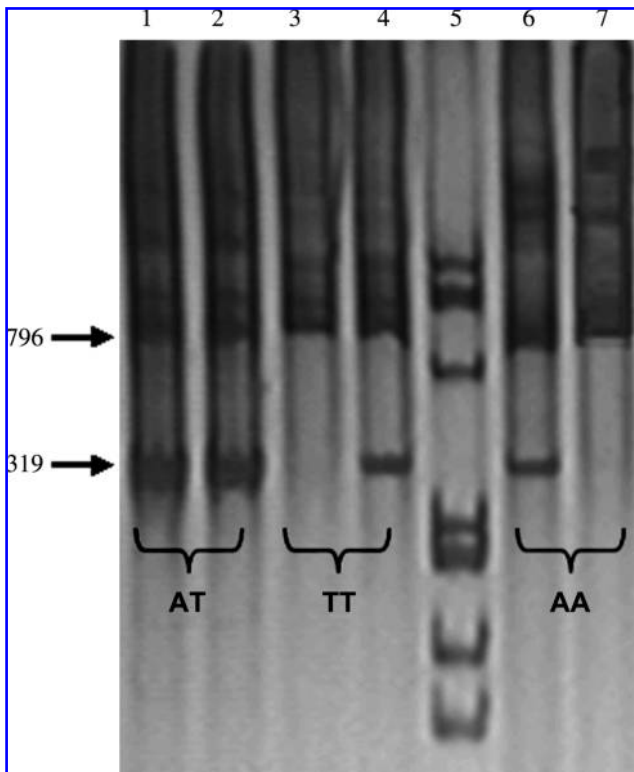
FIG. 1. Polyacrylamide gel (10%) showing the results of sequence-specific primer (SSP)-PCR assay for analyzing the rs4073 polymorphism of the *IL8* gene (319 bp). A control band is present in all lanes (796 bp). Two lanes were necessary to genotype a subject: in the first lane was applied the SSP-PCR to identify the A allele, and in the second lane to identify the T allele. Lane 5: PhiX174 *Hae*III molecular weight marker.

though sex and genotypes of the SNP (rs4073) were not associated with periodontitis, we included them in the multivariate analysis, to adjust for any small confounding effects. The multiple logistic regression analysis also demonstrated that the association between the SNP (rs4073) and periodontitis is not significant, even after adjusting for covariates, including age, sex, skin color, and smoking status. Similarly, focusing on the alleles of the SNP (rs4073) and considering the T allele as a reference, the univariate and multivariate analyses showed that the A allele was not associated with periodontitis (OR = 1.199 & 95% CI: 0.93–1.544, and OR = 1.182 & 95% CI: 0.88–1.577, respectively).

Discussion

Genetic studies in several distinct populations have shown an association between the SNP (rs4073) in the *IL8* gene and inflammatory diseases such as multiple sclerosis, bronchiolitis, and asthma (Hull *et al.*, 2000; Heinzmann *et al.*, 2004; Kamali-Sarvestani *et al.*, 2006). Periodontitis is caused by a bacterial infection in which the periodontal tissues become inflamed and are destroyed by the action of the inflammatory process (Loos *et al.*, 2005). In addition, considering the multifactorial nature of the periodontitis, there is strong supporting evidence that genes play a role in predisposition to PD. In a study with 117 adult twins, Michalowicz *et al.*

(2000) demonstrated that periodontitis had a heritability of approximately 50%, which was unaltered after adjustments for behavioral variables, including smoking. Also, an increasing number of studies, conducted with different ethnic groups, have verified that there is a genetic susceptibility to periodontitis, mainly in genes of the host immune system, such as cytokines (Kornman *et al.*, 1997; Scarel-Caminaga *et al.*, 2002, 2004; Moreira *et al.*, 2007; Wagner *et al.*, 2007). However, in spite of the biological relevance of the *IL8* gene and its participation in the inflammatory reaction of the periodontium (Tamura *et al.*, 1992; Yamamoto *et al.*, 2006), polymorphisms in this gene have never been investigated in association studies involving periodontitis.

In the present study, the functional SNP (rs4073) in the *IL8* gene was not found to be associated with periodontitis. This result indicates that despite the fact that the A allele of the SNP (rs4073) has previously been shown to be associated with higher levels of IL-8 production, and associated with a higher risk of bronchiolitis, atrophic gastritis, and distal gastric cancer (Hull *et al.*, 2000; Rovin *et al.*, 2002; Puthothu *et al.*, 2007), a negative association can be found for a different disease and in a different population. A possible explanation for the negative association of the SNP (rs4073) with periodontitis is that this SNP was analyzed alone. Some studies have shown lack of, or a slight disease association, when the SNP (rs4073) was analyzed alone, but they demonstrated significant association when the SNP (rs4073) was as part of a haplotype that included other nearby SNPs that were in linkage disequilibrium with the SNP (rs4073) (Rovin *et al.*, 2002; Puthothu *et al.*, 2007). Therefore, to analyze the genetic background of disease development, it seems more appropriate to investigate the cooperative influence of other polymorphisms in linkage disequilibrium with a functional SNP (Kinane and Hart, 2003).

To evaluate any potential confounding effects that could cause bias in this association study of periodontitis, important factors known to influence the pathogenesis of periodontitis were assessed by univariate and multivariate analysis. The univariate analysis showed that age, skin color, and smoking status were associated with periodontitis (Table 2). These findings were replicated in the multivariate analysis. Regarding age, an important risk factor for periodontitis, Grossi *et al.* (1995) observed an OR = 2.6 (95% CI: 1.75–3.83) for the age group 35–44 years and an OR = 24.08 (95% CI: 15.93–36.29) for the age group 65–74 years, which are comparable to the results of the present study (Table 2) (Grossi *et al.*, 1995). There is evidence that both the prevalence and severity of periodontitis increase with increasing age (Borrell and Papapanou, 2005). However, this age effect can conceivably represent the cumulative effect of prolonged exposure to other risk factors (Papapanou *et al.*, 1991). Smoking has been established as an important risk factor for periodontitis (Genco, 1996; Kornman *et al.*, 1997; Bergstrom, 2004; Kornman, 2005), which is in agreement with our study (OR$_{adjusted}$ = 4.0; 95% CI: 2.205–7.292). Some studies have shown that smokers had significantly worse clinical symptoms of periodontitis than nonsmokers (Grossi *et al.*, 1995; Okamoto *et al.*, 2006).

In this study, skin color was found to be a confounding factor for periodontitis (OR = 1.69 for lighter-skinned blacks; OR = 2.66 for darker-skinned blacks). The significance of the statistical values for these groups increased in the mul-

tivariate analysis (Table 2). Similar results were found in another study enrolling Brazilian individuals in which lighter-skinned black people and darker-skinned black people presented higher levels of PD when compared with white people (OR = 1.5 & 95% CI: 1.2–1.8, and OR = 1.6 & 95% CI: 1.2–2.1, respectively), even after controlling for covariates like age and sex (Peres *et al.*, 2007).

The main limitation of our study is the lack of information on severity of periodontitis. However, our data were originally produced in a survey context in which the aim of the study was to investigate whether the SNP (rs4073) is associated with periodontitis.

To our knowledge, this is the first report investigating an SNP in the *IL8* gene in relation to periodontitis, in which we concluded that the SNP (rs4073) was not associated with susceptibility to periodontitis in Brazilian individuals, even after controlling for age, sex, skin color, and smoking status. The univariate and multivariate analyses showed that age, skin color, and smoking status were associated with periodontitis.

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Disclosure Statement

No competing financial interests exist.

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