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Association of Haplotypes in the *CXCR2* Gene with Periodontitis in a Brazilian Population

Aline C. Viana,[1] Yeon J. Kim,[1] Karen M.C. Curtis,[2] Rivelto Renzi,[2] Silvana R.P. Orrico,[1] Joni A. Cirelli,[1] and Raquel M. Scarel-Caminaga[2]

CXCR-2 is a receptor of interleukin-8, which is involved in acute and chronic inflammatory processes. Polymorphisms in the *CXCR2* gene have been associated with chronic inflammatory conditions. The aim of this study was to investigate whether the +785(C/T), +1208(T/C), and +1440(G/A) single-nucleotide polymorphisms (SNPs) in the *CXCR2* gene, as well as their haplotypes, are associated with susceptibility to periodontitis in Brazilians. DNA was extracted from the buccal epithelial cells of 487 individuals (control = 215; periodontitis = 272). The SNPs were investigated using the sequence-specific primer–polymerase chain reaction method. Associations between the polymorphisms and subject phenotypes were analyzed using the chi-squared statistical test, followed by univariate and multivariate logistic regression modeling. Haplotypes were reconstructed using the expectation–maximization algorithm, and differences in haplotype distribution between the groups were analyzed to estimate genetic susceptibility for periodontitis development. Univariate and multivariate analysis revealed that age, skin color, and smoking status were associated with periodontitis. The +1440 GG genotype was shown to be protective against periodontitis in both univariate and multivariate analysis (odds ratio $[OR]_{adjusted} = 0.42$; 95% confidence interval [CI] = 0.19, 0.96). A similar relevant result for the +1440 GG was obtained in an alternative analysis considering a subgroup containing only white nonsmokers (OR = 0.37; 95% CI = 0.15, 0.92). White nonsmokers with the CTG/TCG haplotype appeared to be genetically protected against the development of periodontitis (OR = 0.29; 95% CI = 0.09, 0.89), while those carrying the CTG/TCA haplotype were more susceptible to the development of periodontitis (OR = 2.08; 95% CI = 1.24, 3.51). In conclusion, the +1440 SNP and some haplotypes are associated with periodontitis in Brazilian individuals.

Introduction

Periodontitis is a multifactorial disease that is primarily caused by bacterial stimuli (Hafajee and Socransky, 1994) and has a progression closely linked to the host immunoinflammatory response (Yoshie *et al.*, 2007). The inflammatory process in periodontitis is one of the factors responsible for tissue damage, resulting in periodontal pocket formation and destruction of the periodontal ligament and adjacent support bone (Susin *et al.*, 2005; Van Dyke, 2007). Previous studies have reported the importance of specific cytokines, such as interleukin 1 (IL-1) (Massada *et al.*, 1990), IL-2 (Ozawa *et al.*, 2003), IL-4 (Yamazaki *et al.*, 1994), IL-6 (Irwin and Myrillas, 1998), and IL-10 (Yamazaki *et al.*, 1997), in this process.

IL-8, a member of the CXC chemokine family, is primarily responsible for the activation and migration of neutrophils into tissue from peripheral blood (Strieter, 2002). The term CXC is derived from the chemokine structure, which includes two cysteines and one intervening amino acid (Mukaida, 2003). IL-8 is also involved in the initiation and amplification of acute inflammatory reactions and in the chronic inflammatory process (Campa *et al.*, 2005). There are two specific receptors for IL-8, CXCR-1, and CXCR-2 that are responsible for mediating its cellular activities. The amino acid sequences of CXCR-1 and CXCR-2 are 77% identical (Murphy and Tiffany, 1991), and these two receptors are encoded by two single-copy genes located on chromosome 2q34–35 (Morris *et al.*, 1992). CXCR-2 binds IL-8 and other CXC chemokines with high affinity, whereas CXCR-1 binds only IL-8 (Baggiolini, 1998).

The *CXCR2* gene (GenBank accession number M99412) is composed of three exons, and the open reading frame is entirely encoded in the third exon (Sprenger *et al.*, 1994).

Departments of [1]Oral Diagnostics and Surgery and [2]Morphology, School of Dentistry at Araraquara, UNESP—Universidade Estadual Paulista, Araraquara, Brazil.

Some single-nucleotide polymorphisms (SNPs) in the *CXCR2* gene have been reported: +785(C/T) is located in exon 3 (refSNP ID: rs2230054) (reference sequence number from the NCBI's Entrez system) and +1208(T/C) rs1126579 and +1440(G/A) rs1126580 are in the 3′ untranslated region of exon 3 (Renzoni *et al*., 2000).

Associations of these SNPs with chronic inflammatory conditions, especially respiratory and rheumatoid diseases, were confirmed by several studies (Kato *et al*., 2000; Renzoni *et al*., 2000; Brown *et al*., 2006). Renzoni *et al*. (2000) found that individuals homozygous for both +785 C and +1208 T alleles of the *CXCR2* gene were more susceptible to developing systemic sclerosis.

Although the importance of the polymorphisms in the *CXCR2* gene in inflammatory process has been confirmed (Barnes, 1999; Kato *et al*., 2000; Qiu *et al*., 2003), it is not known whether these SNPs could also be related to periodontitis. Therefore, the aim of this study was to investigate whether +785(C/T), +1208(T/C), and +1440(G/A) SNPs in the *CXCR2* gene, as well as their haplotypes, are 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 487 unrelated subjects were recruited from the patient pool of the School of Dentistry at Araraquara, São Paulo State University (UNESP), from November 2004 to 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 of the aims and methods of this study, and all gave their written consent.

The exclusion criteria for enrolling patients in the study were use of prophylactic antibiotics, chronic usage of antiinflammatory drugs, current pregnancy, ongoing orthodontic therapy, and self-declared history of diseases that influence the immune system, diabetes mellitus, HIV infection, or immunosuppressive chemotherapy (Kim *et al*., 2009). Each subject was examined by one of two calibrated periodontists, who carried out the periodontal examinations throughout the study period (weighted kappa = 0.74, considering the probing depth [PD]). The clinical signs and parameters, including PD, clinical attachment loss (CAL), and bleeding on probing, were assessed at six sites around each tooth using a periodontal Williams probe (Trinity, Campo Mourão, Brazil). The subjects were categorized into two groups:

- Control group: subjects exhibiting no sites with CAL and PD $\geq$3 mm and no bleeding on probing
- Periodontitis group: subjects exhibiting one or more sites with CAL and PD $\geq$3 mm and bleeding on probing.

Information on smoking status was obtained using a self-reported questionnaire, and each subject was classified as a "smoker" or "nonsmoker" according to Kornman *et al*. (1997). Smokers were defined as current smokers, and nonsmokers were subjects who had never smoked or who were former smokers who had quit smoking at least 5 years ago.

Analysis of genetic polymorphisms

Buccal epithelial cells from the subjects were obtained with 3 mL of 3% glucose mouthwash for 2 min. DNA was extracted with a phenol/chloroform/isoamyl alcohol (25:24:1) solution and precipitated with a salt ethanol solution (Sambrook, 2001). The +785(C/T), +1208(T/C), and +1440(G/A) SNPs in the *CXCR2* gene were examined using the sequence-specific primer–polymerase chain reaction method (SSP-PCR) as previously reported (Renzoni *et al*., 2000). SSP-PCRs were performed in a 13 μL mixture containing 1×buffer (20 mM Tris-HCl and 50 mM KCl, pH 8.4; Invitrogen, São Paulo, Brazil), 0.2 mM of each dNTP (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom), 2.0 mM $MgCl_2$, 3 U Platinum *Taq* DNA Polymerase (Invitrogen), and 150 ng of genomic DNA. The polymorphisms were amplified using primer sets and thermocycler conditions according to Renzoni *et al*. (2000), with some modifications. For the +1208(T/C) SNP, 0.2 μM of each control APC, forward and reverse primer were used, and the forward primer 5′ AGGCTGGCCAACGGGG/A 3′ was used for the +1440(G/A) SNP, giving a product size of 433 bp.

All of the SSP-PCRs were performed in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany), followed by electrophoresis on a 10% polyacrylamide gel (USB, Cleveland, OH), stained by the silver staining method and photographed using the GDS 8000 System (UVP, Upland, CA).

Statistical analysis

The illustrative power calculations used to estimate 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 the chronic periodontitis prevalence of 0.06 (Dini and Castellanos, 1995).

Differences between the allelic and genotypic frequencies of polymorphisms in the *CXCR2* gene in the control and periodontitis groups were analyzed by the two-sided Fisher's exact test or, in case of a rare polymorphism, by the CLUMP program that employs Monte Carlo simulations (Sham and Curtis, 1995). Associations between the polymorphisms 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 version 9 (SAS Institute, Cary, NC).

The ARLEQUIN version 3.1 program (Excoffier *et al*., 2005) was used to calculate Hardy–Weinberg equilibrium, to reconstruct haplotypes by the expectation–maximization algorithm (as the gametic phase is unknown) and to evaluate a likelihood ratio test of linkage disequilibrium. Differences in the haplotype distributions between the studied groups were assessed by the CLUMP program. The relationship between the computationally inferred haplotypes arranged as alleles or genotypes and periodontal disease susceptibility was analyzed by the two-sided Fisher's exact test and by the odds ratio (OR) and 95% confidence interval (95% CI) calculations using the GraphPad InStat version 3.05 software (San Diego, CA). In a subgroup analysis, smokers were excluded and

statistical analyses were performed in nonsmokers as well. The differences were considered significant when $p < 0.05$.

Results

SNP analysis

The power calculations performed in this study show that the sample size required to ascertain the significance of an association between periodontal disease and the studied genetic polymorphisms with an alpha value of 0.001 and power of 95% was 175 individuals. Therefore, the number of subjects enrolled in this study was large enough to detect association with an acceptable level of confidence.

The population investigated here was primarily composed of female subjects (61.4%), whites (58.3%), and nonsmokers (83.2%) (Table 1). The subjects were classified according to their skin color, as proposed by Peres *et al*. (2007), as whites (predominantly of European heritage), darker-skinned blacks (predominantly African heritage), lighter-skinned blacks (a mixture between European, African, and Amerindian heritages), or yellow (Asian descent) (Table 1).

The control group had an average of 27.6 (±2.1) teeth remaining, while the periodontitis group had 19.7 (±4.3) teeth remaining. The number of teeth with PD and CAL >3 mm was 0 in the control group and 5.65 (±3.6) in the periodontitis group.

In the control group, only the genotype distribution of the +1440 SNP (rs1126580) was consistent with the assumption of Hardy–Weinberg equilibrium. Table 2 shows the results of the univariate analysis used to evaluate any association between periodontitis and the age, sex, skin color, smoking status, and genotypic distributions of the patients. Significant associations were observed with age (ORs ranging from 3.66 for age group 30–39 to 29.2 for age group 60–69), skin color (OR = 1.78 for lighter-skinned blacks; OR = 2.57 for darker-skinned blacks), smoking status (OR = 3.36), the SNP rs1126580 (OR = 0.4 for GG genotype; OR = 1.605 for GA genotype), and periodontitis. Therefore, these characteristics were considered confounding factors of periodontitis. In contrast, neither sex nor the +785 (rs2230054) or +1208 (rs1126579) SNPs in the *CXCR2* gene were associated with periodontitis (Table 2).

Table 1. Characteristics of the Studied Groups

	Control (n = *215*)	*Periodontitis* (n = *272*)	*Total* (n = *487*)
Age, mean (±SD), years	35.3 (±10.4)	43.4 (±10.5)	39.7 (±11.2)
Sex, *n* (%)			
Female	127 (59)	172 (63.2)	299 (61.4)
Male	88 (41)	100 (36.8)	188 (38.6)
Skin color, *n* (%)			
White	144 (67)	140 (51.4)	284 (58.3)
Darker-skinned blacks	24 (11.2)	60 (22)	84 (17.2)
Lighter-skinned blacks	41 (19)	71 (26)	112 (23)
Yellow	6 (2.8)	1 (0.6)	7 (1.5)
Smoking habits, *n* (%)			
Nonsmokers	197 (91.6)	208 (76.4)	405 (83.2)
Smokers	18 (8.4)	64 (23.6)	82 (16.8)

To more accurately evaluate the strength of any association and to eliminate the distortion caused by confounding effects, multivariate analysis was performed. Although sex and the rs2230054 and rs1126579 SNPs were not associated with periodontitis, we included them in the multivariate analysis to adjust for any small confounding effects. Except for the GG genotype of the +1440 SNP (rs1126580), all of the confounding factors detected by univariate analysis were also detected by multivariate analysis (Table 2).

In the univariate analysis, for the +1440 SNP, when comparing the three genotypes, it was observed that the GG genotype was shown to be protective against periodontitis (OR = 0.4; CI = 0.193, 0.831) (Table 2); when comparing the GG with GA and AA, the GG genotype was also found to be protective against periodontitis ($p = 0.0002$; OR = 0.287; 95% CI = 0.146, 0.562). Multiple logistic regression analysis confirmed that individuals carrying the GG genotype of the rs1126580 SNP were protected against the development of periodontitis (OR = 0.423; 95% CI = 0.187, 0.957), even after adjusting for covariates, including age, sex, skin color, and smoking status.

Indeed, the data in Table 3, which shows the allele and genotype frequencies of the studied SNPs in different subgroups, demonstrated that the +1440 SNP genotype distribution was significantly different for all subgroups: total ($p < 0.0001$), nonsmokers ($p = 0.0002$), whites ($p = 0.0067$), and white nonsmokers ($p = 0.0139$), where all the OR and CI values indicated the GG genotype as associated with protection against periodontitis. For example, considering a subgroup formed by only white nonsmokers, the +1440 GG showed the OR = 0.37 and 95% CI = 0.15, 0.92. These significant results in Table 3 are in agreement with the results obtained in the univariate and multivariate analysis (Table 2), in which the GG genotype of the rs1126580 SNP seems to protect individuals against periodontitis.

Haplotype analysis

In the control and periodontitis groups, linkage disequilibrium was observed among nearly all SNPs ($p = 0.00000 \pm 0.00000$). The exception to this is that linkage disequilibrium was not detected between the rs2230054 and rs1126580 SNPs ($p = 0.34604 \pm 0.0046$) in the control group.

The distributions of the haplotype sets in the control and periodontitis groups were examined using the CLUMP program. As summarized in Table 4, this analysis revealed a significant difference between the two groups in both the total sample ($p = 0.0029$), nonsmokers and white nonsmokers ($p = 0.0009$). When each haplotype was evaluated for an association with periodontitis in the white nonsmoker subgroup, we observed that individuals carrying the haplotypes TCA (OR = 1.47; 95% CI = 1.01, 2.14) and CCG (OR = 6.13; 95% CI = 1.32, 28.3) were more likely to develop periodontitis. The haplotypes CCA (OR = 0.41; 95% CI = 0.19, 0.87) and TCG (OR = 0.33; 95% CI = 0.15, 0.73) appeared to have the opposite effect; that is, these haplotypes seemed to protect individuals against the disease (Table 4).

Computationally obtained maximum-likelihood haplotype frequencies revealed that the distributions of the haplotypes arranged as genotypes (assessed by the CLUMP

Table 2. Odds Ratios for Periodontitis and Characteristics of the Patients (Including the Genotypes of the rs2230054, rs1126579, and rs1126580 Single-Nucleotide Polymorphisms in the *CXCR2* Gene)

Characteristics of patients		*Control,* n *(%)*	*Periodontitis,* n *(%)*	p	*Univariate OR (95% CI)*	*Multivariate OR (95% CI)*
Total		215	272			
Age	20–29	80 (75.47)	26 (24.53)		Reference	Reference
	30–39	63 (45.65)	75 (54.35)		3.663 (2.103, 6.380)	3.333 (1.786, 6.217)
	40–49	53 (33.97)	103 (66.03)	<0.0001	5.980 (3.440, 10.393)	6.567 (3.544, 12.170)
	50–59	15 (25.00)	45 (75.00)		9.231 (4.435, 19.211)	10.834 (4.744, 24.745)
	60–69	2 (9.52)	19 (90.48)		29.230 (6.376, 134.011)	38.288 (7.767, 188.749)
	>70	2 (33.33)	4 (66.67)		6.154 (1.065, 35.558)	11.493 (1.144, 115.515)
Sex	Male	88 (46.81)	100 (53.19)	0.3484	Reference	Reference
	Female	127 (42.47)	172 (57.53)		1.192 (0.826, 1.720)	1.045 (0.667, 1.638)
Skin color	White	144 (50.70)	140 (49.30)		Reference	Reference
	Darker-skinned blacks	24 (28.57)	60 (71.43)		2.571 (1.517, 4.358)	2.488 (1.357, 4.560)
	Lighter-skinned blacks	41 (36.61)	71 (63.39)	0.0001	1.781 (1.136, 2.792)	2.400 (1.416, 4.068)
	Yellow	6 (85.71)	1 (14.29)		0.172 (0.020, 1.442)	0.327 (0.032, 3.365)
Smoking habits	Nonsmokers	197 (48.64)	208 (51.36)	<0.0001	Reference	Reference
	Smokers	18 (21.95)	64 (78.05)		3.368 (1.927, 5.884)	3.597 (1.910, 6.772)
SNP rs2230054 (alias +785)	TT	5 (38.46)	8 (61.54)		Reference	Reference
	TC	193 (44.16)	244 (55.84)	0.896	0.790 (0.254, 2.454)	0.882 (0.242, 3.219)
	CC	17 (45.95)	20 (54.05)		0.735 (0.202, 2.674)	0.709 (0.163, 3.091)
	T	203	260	0.907	Reference	Reference
	C	227	284		0.982 (0.762, 1.265)	0.965 (0.724, 1.284)
SNPrs1126579 (alias +1208)	TT	21 (40.38)	31 (59.62)		0.824 (0.391, 1.737)	0.564 (0.305, 1.042)
	TC	170 (46.20)	198 (53.80)	0.2455	0.650 (0.379, 1.115)	0.857 (0.356, 2.062)
	CC	24 (35.82)	43 (64.18)		Reference	Reference
	T	212	260	0.687	0.95 (0.738, 1.224)	0.944 (0.709, 1.256)
	C	218	284		Reference	Reference
SNPrs1126580 (alias +1440)	GG	32 (71.11)	13 (28.89)		0.400 (0.193, 0.831)	0.423 (0.187, 0.957)
	GA	119 (38.02)	194 (61.98)	<0.0001	1.605 (1.061, 2.428)	1.469 (0.915, 2.358)
	AA	64 (49.61)	65 (50.39)		Reference	Reference
	G	183	220	0.548	0.925 (0.716, 1.196)	0.910 (0.681, 1.217)
	A	247	324		Reference	Reference

p-Values of alleles were calculated by Fischer's exact test, *p*-values of genotypes, age, sex, skin color, and smoke habits were calculated by chi-squared test.
SNPs, single-nucleotide polymorphisms; OR (95% CI), odds ratio values with their respective 95% of confidence intervals.

Table 3. Alleles and Genotype Distribution of the *CXCR2* Single-Nucleotide Polymorphisms in the Studied Individuals

	Total			*Nonsmokers*			*Whites*			*White nonsmokers*		
SNP	*Control*, n *(%)*	*DP*, n *(%)*	p	*Control*, n *(%)*	*DP*, n *(%)*	p	*Control*, n *(%)*	*DP*, n *(%)*	p	*Control*, n *(%)*	*DP*, n *(%)*	p
+785												
Allele	$n=430$	$n=544$	0.8972	$n=394$	$n=416$	0.7250	$n=288$	$n=280$	1.000	$n=262$	$n=222$	0.9275
T	203 (47.2)	260 (48.0)		184 (46.7)	200 (48.0)		137 (47.6)	134 (47.9)		124 (47.3)	106 (47.7)	
C	227 (52.8)	284 (52.0)		210 (53.3)	216 (52.0)		151 (52.4)	146 (52.1)		138 (52.7)	116 (52.3)	
Genotype	$n=215$	$n=272$	0.8963	$n=197$	$n=208$	0.6309	$n=144$	$n=140$	0.653	$n=131$	$n=111$	0.5373
TT	5 (2.3)	8 (3.0)		4 (2.0)	7 (3.4)		2 (1.4)	4 (2.9)		2 (1.6)	4 (3.6)	
TC	193 (89.8)	244 (89.7)		176 (89.4)	186 (89.4)		133 (92.4)	126 (90.0)		120 (91.6)	98 (88.3)	
CC	17 (7.9)	20 (7.3)		17 (8.6)	15 (7.2)		9 (6.2)	10 (7.1)		9 (6.8)	9 (8.1)	
+1208												
Allele	$n=430$	$n=544$	0.6517	$n=394$	$n=416$	0.7785	$n=288$	$n=280$	0.5022	$n=262$	$n=222$	0.7843
T	212 (49.3)	260 (47.8)		193 (49.0)	199 (47.8)		140 (48.6)	128 (45.7)		128 (48.8)	105 (47.3)	
C	218 (50.7)	284 (52.2)		201 (51.0)	217 (52.2)		148 (51.4)	152 (54.3)		134 (51.2)	117 (52.7)	
Genotype	$n=215$	$n=272$	0.2455	$n=197$	$n=208$	0.3461	$n=144$	$n=140$	0.2656	$n=131$	$n=111$	0.6171
TT	21 (9.8)	31 (11.4)		19 (9.6)	24 (11.5)		15 (10.4)	16 (11.4)		14 (10.8)	13 (11.7)	
TC	170 (79.0)	198 (72.8)		155 (78.8)	151 (72.6)		110 (76.4)	96 (68.6)		100 (76.3)	79 (71.2)	
CC	24 (11.2)	43 (15.8)		23 (11.6)	33 (15.9)		19 (13.2)	28 (20.0)		17 (12.9)	19 (17.1)	
+1440												
Allele	$n=430$	$n=544$	0.5130	$n=394$	$n=416$	0.8312	$n=288$	$n=280$	0.6706	$n=262$	$n=222$	0.9267
G	183 (42.6)	220 (40.4)		168 (42.6)	174 (41.8)		123 (42.7)	114 (40.7)		111 (42.4)	93 (41.9)	
A	247 (57.4)	324 (59.6)		226 (57.4)	242 (58.2)		165 (57.3)	166 (59.3)		151 (57.6)	129 (58.1)	
Genotype	$n=215$	$n=272$	**<0.0001**	$n=197$	$n=208$	**0.0002**	$n=144$	$n=140$	**0.0067**	$n=131$	$n=111$	**0.0139**
GG	32 (14.9)	13 (4.8)		31 (15.7)	12 (5.8)		21 (14.5)	7 (5.0)		20 (15.3)	7 (6.3)	
GA	119 (55.3)	194 (71.3)		106 (53.9)	150 (72.1)		81 (56.3)	100 (71.4)		71 (54.2)	79 (71.2)	
AA	64 (29.8)	65 (23.9)		60 (30.4)	46 (22.1)		42 (29.2)	33 (23.6)		40 (30.5)	25 (22.5)	
GG	32	13	**0.0002**	31	12	**0.0012**	21	7	**0.009**	20	7	**0.039**
GA+AA	183	259		166	196		123	133		111	104	
	OR = 0.287; 95% CI = 0.146, 0.562			OR = 0.328; 95% CI = 0.163, 0.659			OR = 0.308; 95% CI = 0.127, 0.751			OR = 0.374; 95% CI = 0.152, 0.920		

p-values of alleles were calculated by Fischer's exact test and genotypes by chi-squared test. Statistically significant *p*-values are presented in bold.
DP, periodontitis group.

Table 4. Distribution of *CXCR2* Haplotypes Found in the Studied Groups

Haplotypes	*Total*					*Nonsmokers*					*White nonsmokers*				
+785 +1208 +1440	*Control, n = 430 (%)*	*Periodontitis, n = 544 (%)*	p	*OR*	*95% CI*	*Control, n = 334 (%)*	*Periodontitis, n = 322 (%)*	p	*OR*	*95% CI*	*Control, n = 262 (%)*	*Periodontitis, n = 222 (%)*	p	*OR*	*95% CI*
TCA	135 (31.4)	203 (37.3)	**0.057**	**1.12**	**1.00, 1.25**	121 (30.7)	160 (38.5)	**0.0005**[a]	**1.32**	**1.13, 1.54**	79 (30.2)	86 (38.7)	**0.05**	**1.47**	**1.01, 2.14**
CTG	123 (28.6)	166 (30.5)	0.52	1.0	0.92, 1.17	111 (28.2)	133 (32.0)	**0.036**	**1.19**	**1.02, 1.39**	72 (27.5)	72 (32.4)	0.272	1.27	0.86, 1.87
CTA	58 (13.5)	58 (10.7)	0.195	0.88	0.72, 1.07	55 (14.0)	43 (10.3)	0.275	0.88	0.69, 1.11	37 (14.1)	22 (9.9)	0.166	0.67	0.38, 1.17
CCA	38 (8.8)	33 (6.0)	0.107	0.82	0.64, 1.06	36 (9.1)	19 (4.6)	**0.025**	**0.68**	**0.47, 0.99**	27 (10.3)	10 (4.5)	**0.017**	**0.41**	**0.19, 0.87**
TCG	37 (8.6)	20 (3.7)	**0.0014**[a]	**0.61**	**0.43, 0.88**	36 (9.1)	17 (4.1)	**0.010**	**0.63**	**0.43, 0.95**	27 (10.3)	8 (3.6)	**0.004**	**0.33**	**0.15, 0.73**
TTA	16 (3.7)	27 (5.0)	0.433	1.13	0.89, 1.43	14 (3.6)	16 (3.8)	0.710	1.09	0.77, 1.54	8 (3.1)	8 (3.6)	0.802	1.18	0.44, 3.22
TTG	15 (3.5)	10 (1.8)	0.152	0.71	0.44, 1.15	13 (3.3)	8 (1.9)	0.377	0.77	0.44, 1.34	10 (3.8)	6 (2.7)	0.613	0.70	0.25, 1.96
CCG	8 (1.9)	27 (5.0)	**0.0094**	**1.40**	**1.16, 1.69**	8 (2.0)	20 (4.8)	**0.019**	**1.49**	**1.16, 1.90**	2 (0.8)	10 (4.5)	**0.015**	**6.13**	**1.32, 28.3**
p[b]	**0.0029**[a,b]					**0.0009**[a,b]					**0.0009**[a,b]				

Statistically significant values are presented in bold; p-values not marked were calculated by Fisher exact test.
[a]Statistically significant results after Bonferroni correction for multiple tests.
[b]p-values obtained from the T4 values of CLUMP program.

program) were significantly different between the control and periodontitis groups for both the total sample ($p = 0.0069$), nonsmokers ($p = 0.0019$) and white nonsmokers ($p = 0.0149$) (Table 5). Analysis of each haplotype indicated that nonsmokers carrying the CTG/TCA (OR = 2.08; 95% CI = 1.24, 3.51) were more susceptible to the development of periodontitis than individuals carrying other haplotypes. White nonsmokers with the CTG/TCG genotype appeared to be genetically protected against the development of periodontitis (OR = 0.29; 95% CI = 0.09, 0.89) (Table 5).

Discussion

Periodontitis has a complex etiology, as it is primarily caused by bacteria (Van Dyke, 2007), but is also affected by several risk factors, including smoking habits (Grossi *et al.*, 1994), diabetes (Cairo *et al.*, 2001), and genetic polymorphisms (Schenkein, 2002). Previous studies have suggested that inflammatory cytokines also contribute to periodontitis pathogenesis (Page and Schroeder, 1976; Kornman *et al.*, 1997; Graves, 1999). Thus, it is reasonable to assume that genetic variations, such as SNPs, in cytokines and cytokine receptor genes (Breunis *et al.*, 2007) may contribute to susceptibility to periodontitis.

The results presented here revealed that individuals carrying the GA genotype at the +1440 SNP (rs1126580) were protected against the development of periodontitis, even after adjusting for covariates (Table 2). A similar protector effect against the development of Classic Kaposi Sarcoma (Brown *et al.*, 2006) was observed with the presence of both the +1440G SNP and the +1208T SNP (rs1126579). Classic Kaposi Sarcoma is an inflammatory-mediated disease characterized by localized pathogenesis and involves the expression of proinflammatory cytokines (Miles *et al.*, 1990). The +1208(T/C) and +1440(G/A) genetic variants in the *CXCR2* gene are located within the 3′ untranslated region of exon 3 and have the capacity to alter mRNA processing, stability or translation (Ahuja *et al.*, 1994; Sprenger *et al.*, 1994). Functional studies suggest that *CXCR2* indirectly activates fibroblasts by mediating the recruitment of T cells (Santamaria *et al.*, 1996), a process similar to that observed both in Kaposi Sarcoma spindle cell transformation (Brown *et al.*, 2006) and in periodontitis (Van Dyke, 2007). Unfortunately, studies investigating the functionality of polymorphisms in the *CXCR2* gene have not been performed. It is unclear, for example, whether a polymorphism in this gene could influence the expression level of the CXCR-2 protein or whether an SNP could modify the capacity of the receptor to bind to IL-8.

The excess heterozygotes relative to the expected number of heterozygotes for all SNPs may explain why Hardy–Weinberg equilibrium of the genotype distributions in our study was not observed. Indeed, high heterozygosity was previously observed when approximately 50% of the individuals were genotyped for the +1440 SNP by the PCR–restriction fragment length polymorphism method (Viana *et al.*, 2007). Errors in genotyping are commonly used to explain departures from Hardy–Weinberg equilibrium. In this study, however, we believe that this is not a strong possibility because when a sample demonstrated an uncertain pattern of bands, it was repeated until an accurate genotype could be obtained. Another possible explanation

Table 5. Distribution of *CXCR2* Haplotypes (Arranged as Genotypes) in the Studied Groups

Genotypes	*Total*					*Nonsmokers*					*White nonsmokers*				
+785 +1208 +1440/ +785 +1208 +1440	*Control, n = 215 (%)*	*Periodontitis, n = 272 (%)*	p	*OR*	*95% CI*	*Control, n = 197 (%)*	*Periodontitis, n = 208 (%)*	p	*OR*	*95% CI*	*Control, n = 131 (%)*	*Periodontitis, n = 111 (%)*	p	*OR*	*95% CI*
CTG/TCA	75 (34.8)	123 (45.2)	**0.026**	**1.21**	**1.03, 1.41**	66 (33.5)	100 (48.1)	**0.003**	**1.33**	**1.11, 1.61**	43 (32.8)	56 (50.5)	**0.006**	**2.08**	**1.24, 3.51**
TCA/CTA	38 (17.7)	38 (14.0)	0.314	0.87	0.69, 1.12	35 (17.8)	30 (14.4)	0.417	0.88	0.66, 1.17	22 (16.8)	15 (13.5)	0.591	0.77	0.38, 1.58
CTG/TCG	22 (10.2)	9 (3.3)	**0.002**[a]	**0.5**	**0.29, 0.88**	21 (10.7)	8 (3.8)	**0.011**	**0.52**	**0.28, 0.94**	15 (11.5)	4 (3.6)	**0.029**	**0.29**	**0.09, 0.89**
CTA/TCG	10 (4.7)	4 (1.5)	0.053	0.5	0.22, 1.59	9 (4.7)	3 (1.4)	0.080	0.48	0.18, 1.28	8 (6.1)	2 (1.8)	0.114	0.28	0.06, 1.36
CTG/CCA	9 (4.2)	9 (3.3)	0.635	0.89	0.56, 1.43	9 (4.7)	7 (3.4)	0.615	0.85	0.48, 1.48	6 (4.6)	3 (2.7)	0.513	0.58	0.14, 2.37
CTG/TTA	8 (3.7)	17 (6.3)	0.223	1.23	0.93, 1.63	6 (3.0)	9 (4.3)	0.602	1.17	0.77, 1.80	3 (2.3)	4 (3.6)	0.706	1.59	0.35, 7.28
TTG/CTG	5 (2.3)	3 (1.1)	0.31	0.66	0.27, 1.64	5 (2.5)	3 (1.4)	0.490	0.73	0.29, 1.79	4 (3.1)	2 (1.8)	0.69	0.58	0.10, 3.24
TTA/CCA	3 (1.4)	3 (1.1)	1.000	0.89	0.40, 2.00	3 (1.5)	1 (0.5)	0.360	0.48	0.09, 2.65	2 (1.5)	1 (0.9)	1.00	0.59	0.05, 6.56
CTA/TTG	3 (1.4)	2 (0.7)	0.66	0.71	0.24, 2.10	3 (1.5)	2 (1.0)	0.680	0.77	0.26, 2.28	2 (1.5)	1 (0.9)	1.00	0.59	0.05, 6.56
TTA/CTA	3 (1.4)	3 (1.1)	1.000	0.89	0.40, 2.00	3 (1.5)	1 (0.5)	0.360	0.48	0.09, 2.65	2 (1.5)	0	0.50	[b]	
TCA/CCG	2 (0.9)	20 (7.4)	**0.0006**[a]	**1.68**	**1.43, 1.96**	2 (1.0)	15 (7.2)	**0.002**[a]	**1.77**	**1.45, 2.17**	1 (0.8)	6 (5.4)	0.07	7.43	0.88, 62.7
TCG/CCA	2 (0.9)	2 (0.7)	1.000	0.89	0.33, 2.39	2 (1.0)	1 (0.5)	0.610	0.65	0.13, 3.22	2 (1.5)	1 (0.9)	1.00	0.59	0.05, 6.56
TCA/CCA	15 (6.9)	15 (5.5)	0.570	0.89	0.62, 1.28	14 (7.1)	8 (3.8)	0.190	0.69	0.40, 1.22	11 (8.4)	4 (3.6)	0.18	0.41	0.13, 1.32
TTG/TCA	2 (0.9)	3 (1.1)	1.000	0.89	0.33, 2.39	2 (1.0)	3 (1.4)	1.000	1.17	0.57, 2.41	1 (0.8)	2 (1.8)	0.59	2.38	0.21, 26.68
CCA/TTG	2 (0.9)	1 (0.4)	0.580	0.59	0.12, 2.96	2 (1.0)	1 (0.5)	0.610	0.65	0.13, 3.22	2 (1.5)	0	0.50	[b]	
CTA/CCA	2 (0.9)	2 (0.7)	1.000	0.89	0.33, 2.39	2 (1.0)	0	0.24	[b]		2 (1.5)	0	0.50	[b]	
CTA/CCG	1 (0.5)	2 (0.7)	1.000	1.19	0.53, 2.67	1 (0.5)	2 (1.0)	1.000	1.30	0.58, 2.91	0	1 (0.9)	0.46	[b]	
CCG/TTA	1 (0.5)	2 (0.7)	1.000	1.19	0.53, 2.67	1 (0.5)	2 (1.0)	1.000	1.30	0.58, 2.91	0	1 (0.9)	0.46	[b]	
CCG/TTG	1 (0.5)	1 (0.4)	1.000	0.89	0.22, 3.58	1 (0.5)	1 (0.5)	1.000	0.97	0.24, 3.91	0	1 (0.9)	0.46	[b]	
TTA/TCG	1 (0.5)	1 (0.4)	1.000	0.89	0.22, 3.58	0	0		[b]		0	0		[b]	
CCA/CCA	2 (0.9)	0	0.190	[b]		2 (1.0)	0	0.236	[b]		1 (0.8)	0	1.00	[b]	
CTG/CCG	3 (1.4)	0	0.080	[b]		3 (1.5)	0	0.114	[b]		0	0		[b]	
CTG/CTA	1 (0.5)	6 (2.2)	0.140	1.55	1.13, 2.12	1 (0.5)	5 (2.4)	0.216	1.64	1.13, 2.36	1 (0.8)	3 (2.7)	0.34	3.61	0.37, 35.24
TCG/CCG	1 (0.5)	0	0.440	[b]		1 (0.5)	0	0.486	[b]		1 (0.8)	0	1.00	[b]	
TCG/TCA	1 (0.5)	3 (1.1)	0.630	1.34	0.76, 2.39	1 (0.5)	3 (1.4)	0.623	1.46	0.83, 2.60	1 (0.8)	1 (0.9)	1.00	1.18	0.07, 19.13
TCA/TCA	1 (0.5)	0	0.440	[b]		1 (0.5)	0	0.486	[b]		0	0		[b]	
TTG/TTA	1 (0.5)	0	0.440	[b]		1 (0.5)	0	0.486	[b]		1 (0.8)	0	1.00	[b]	
TTA/TCA	0	2 (0.7)	0.510	[b]		0	2 (1.0)	0.499	[b]		0	2 (1.8)	0.50	[b]	
CCG/CCA	0	1 (0.4)	1.000	[b]		0	1 (0.5)	0.486	[b]		0	1 (0.9)	0.46	[b]	
p	**0.0069**[c]					**0.0019**[c]					**0.0149**[c]				

Statistically significant values are presented in bold; *p*-values not marked were calculated by Fisher's exact test.
[a]Statistically significant results after Bonferroni correction for multiple tests.
[b]OR not calculated because of the presence of zero.
[c]*p*-values obtained from the T4 values in the CLUMP program.

for the departures from Hardy–Weinberg equilibrium is the selection criteria used for patients with periodontitis. Similar findings were obtained in a study focusing on aggressive periodontitis and the −590 and −34 SNPs in the *IL4* gene (Gonzales *et al*., 2007).

When the three SNPs were analyzed together as haplotypes, we found a significant association between the haplotypes and periodontitis susceptibility (Tables 4 and 5). Similar findings were observed with the same population in a previous study (Kim *et al*., 2009) and in other studies of Brazilian individuals in which *IL10* (Scarel-Caminaga *et al*., 2004) and a vitamin D receptor (de Brito Junior *et al*., 2004) were examined. These previous studies corroborate the idea that haplotypes are more powerful in detecting susceptibility alleles than individual polymorphisms and that they may give more information on the disease.

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 also obtained in the multivariate analysis. Similar results were also obtained in a previous study investigating the association of a polymorphism in the *IL8* gene with periodontitis in the same population (Kim *et al*., 2009). It has been established that smoking habits are an important risk factor for the initiation and progression of periodontitis (Genco, 1996; Kornman, 2005; Gonzales *et al*., 2007). Kornman *et al*. (1997) suggested that the smoking-related risk could often obscure the polymorphism-related risk. In the present study, smoking status was a confounding factor of periodontitis ($p < 0.0001$) (Table 2). The results of the genetic analyses of the total sample were not notably different from those obtained for nonsmokers only (Tables 3–5). A possible reason for this could be the low frequency of smokers (16.8%) in the total sample. Nevertheless, because others and we have found an association of smoking habits with periodontitis ($OR_{adjusted} = 3.597$; 95% CI = 1.910, 6.772; Table 2), we performed the haplotype analysis without smokers. Without the influence of smoking habits, results of the haplotype analyses showed significant results (Tables 4 and 5). Further, to eliminate as many confounding factors as possible, that is, skin color and smoking habits, all the statistical analyses were performed, including only white individuals who were nonsmokers (Tables 3–5). For white nonsmokers it was found that the CTG/TCA is associated with a genetic predisposition to periodontitis and that the CTG/TCG genotype is associated with protection against periodontitis (Table 5).

These results, however, could be influenced by age. The increase of age has been associated with the prevalence, extent, and severity of periodontitis (Heitz-Mayfield, 2005). It has also been proposed that the increased level of periodontal destruction observed with aging is a result of cumulative destruction rather than increased rates of destruction (Genco, 1996). Therefore, for multivariate analysis to completely eliminate the effect of age on the studied population, the mean age of the subjects should have been similar. Thus, the mean age difference between groups may be a limitation of the present study.

The analysis of haplotypes arranged as alleles revealed a significant difference between their distributions in the control and periodontitis groups, where the TCA and CCG haplotypes were associated with susceptibility to periodontitis and the CCA and TCG haplotypes were associated with protection against the development of periodontitis (Table 4). In the literature, the only study that investigated the same SNPs in the *CXCR2* gene was conducted in a Chinese population. That study found five prevalent haplotypes, with the CTG haplotype being the most predominant, followed by the TCA haplotype (23%) (Hsing *et al*., 2008). In the Brazilian population studied here, eight haplotypes were found; three of these haplotypes (CTA, TTA, and TTG) were identified for the first time in this study. Interestingly, the most common haplotype in the Chinese population, CTG (64.4% for controls), was much less common in the Brazilian population (28.6% for total controls, Table 4) and was associated with susceptibility to periodontitis in Brazilian nonsmokers. The CCA haplotype, which was associated with protection against the development of periodontitis in nonsmokers, was seven times more frequent in Brazilian population (8.8%) than in the Chinese population (1.2%, the rarest haplotype observed by Hsing *et al*., 2008).

Because the significant results of the *CXCR2* gene association with periodontitis were obtained in a Brazilian admixture population, additional studies enrolling other ethnically diverse populations must be performed to ascertain whether this gene could be a marker for periodontitis or other diseases, such as inflammatory diseases. Also, to better understand the results of the association-disease studies, additional studies investigating the functionality of the *CXCR2* polymorphisms will be necessary.

In conclusion, to our knowledge, our findings are the first to indicate an association between the +1440 SNP and haplotypes in the *CXCR2* gene with susceptibility to or protection against periodontitis in Brazilian individuals.

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No competing financial interests exist.

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Address correspondence to:
Raquel M. Scarel-Caminaga, Ph.D.
Department of Morphology
School of Dentistry at Araraquara
UNESP-São Paulo State University, CP 331
Araraquara CEP 14801-903, SP
Brazil

E-mail: raquel@foar.unesp.br

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