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GENETIC ANALYSIS OF THE *IL8* GENE POLYMORPHISM (rs4073) IN GENERALIZED AGGRESSIVE PERIODONTITIS

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Abstract

Objectives—Interleukin (IL)-8 is an important chemokine for regulation of the inflammatory response. A single nucleotide polymorphism (SNP) reference sequence (rs) 4073 in the *IL8* gene has been shown to regulate IL-8 levels after stimulation with lipopolysaccharide. This study investigates the transmission pattern of the *IL8* rs4073 risk allele A and its association with susceptibility to Aggressive Periodontitis (AgP) in families and in a case-control cohort of unrelated individuals from a Brazilian population.

Design—Genotyping was performed by standard polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP) in 13 nuclear families and 184 unrelated subjects. Statistical analysis was performed using the transmission disequilibrium test (TDT) for the family dataset and chi-square test and multivariate logistic regression modeling for the case-control dataset.

Results—TDT analyses did not detect evidence of over transmission of *IL8* rs4073 alleles in affected and unaffected family members (allele T: 52%; allele A: 48%; $p = 0.2252$). How expected, analyses of cases and unrelated controls showed a significant and inverse association of age with AgP; however, a lack of association between genotypes, ethnic groups and generalized AgP was observed.

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Conclusions—The SNP (rs4073) was not associated with AgP in unrelated individuals and there is no evidence of over transmission of the alleles in families with AgP, from Brazilian individuals.

Keywords

aggressive periodontitis; Interleukin-8; single nucleotide; polymorphism; genotype; alleles

Introduction

Aggressive periodontitis (AgP) is a type of periodontitis that can promote a rapid attachment loss and bone destruction that usually affects healthy juveniles or early adults¹ and is characterized by familial aggregation.² Systemic diseases, microbiologic and environmental factors (e.g., smoking, oral hygiene, stress, and life style) have been suggested to influence the risk of developing AgP.³ However, the observed familial aggregation of cases indicates that genetic factors may be critical determinants in disease susceptibility.² Family history is now considered a primary diagnostic criterion for current classification of AgP^{1,4} and family screening could be used as a preventive diagnostic tool.⁴

The gene of interleukin-8, located on chromosome 4q12-q13, encodes the IL8 protein that is the most potent chemokine known and responsible for inducing chemotaxis, which is the directed migration of cells to a site of inflammation.⁵ This chemokine is important in the regulation of the inflammatory response for its ability to recruit and activate acute inflammatory cells. It is also able to mediate the activation and migration of neutrophils, which is the first line of defense against periodontopathic bacteria, from peripheral blood into the tissues.⁶ Moreover, IL-8 is unique as it may be produced early in the inflammatory response, and its presence will persist for a prolonged period of time.⁷

The genetic factors for AgP have been extensively studied in the last decades, including studies aimed to identify candidate genes in association with AgP.^{4,8–13} A polymorphism at position –251 T/A (rs4073) in the *IL8* gene promoter has been described in the literature and associated with several diseases such as gastric cancer, bronchiolitis, breast cancer, macular degeneration and lung cancer.^{14–19} However, the association of this polymorphism with AgP has not yet been investigated. Analyses have shown that the risk A allele up regulates IL-8 levels after stimulation with lipopolysaccharides.¹⁵ Therefore, it would be interesting to investigate the association of the –251 T/A polymorphism (rs4073) with AgP. The intent of this study was to investigate the association of the *IL8* rs4073 polymorphism with generalized AgP in families and unrelated individuals with and without AgP from Brazil.

Material and Methods

Study population

Table 1 summarizes the demographic and clinical characteristics of the families and unrelated individuals in the study. Subjects from the Southeastern region of Brazil were recruited from the patient pool of the Dental Clinics of the School of Dentistry at Piracicaba, State University of Campinas, during the period of July 2008 to July 2009. This study was

approved by the local Institutional Review Board (Protocol number 095/2007), and informed consent was signed by each subject after explanations were provided. All patients received complete intraoral and periodontal examination, including full-mouth plaque index, according to Ainamo and Bay,²⁰ and full-mouth bleeding score, according to Mühlemann and Son,²¹ tooth mobility, radiographic examination, and complete medical and dental questionnaires. Subjects did not have any of the following exclusion criterion: smoking, diseases of the oral hard or soft tissues (except caries and periodontitis), use of orthodontic appliances, need for pre-medication for dental treatment, chronic usage of anti-inflammatory drugs, history of diabetes, hepatitis or HIV infection, immunosuppressive chemotherapy, history of any disease known to severely compromise immune function, presence of acute necrotizing ulcerative gingivitis, or current pregnancy or lactation.

The criteria for inclusion in the study were (i) diagnosis and classification of aggressive periodontitis according to the 1999 Consensus Classification of Periodontal Diseases,¹ (ii) good general healthy, (iii) diagnosis of generalized aggressive periodontitis was characterized by the presence of periodontal pockets with clinical attachment loss and radiographic bone loss in at least 3 teeth (excluding 1st molars and incisors) and at least eight teeth with probing pocket depth (PPD) greater than 5mm and bleeding following pocket probing. At least two of the eight qualifying teeth must have PPD > 7 mm, (iv) subjects younger than 35 years, (v) 20 teeth in both jaws (excluding third molars).

Measurements of probing depth and attachment levels were recorded at six points around each tooth. The individuals of the control group were also examined for the same clinical parameters used in the AgP group, but the subjects did not show any sites with clinical attachment loss and probing pocket depth ≥ 3 mm and presented full-mouth bleeding score (FMBS) < 25%; tooth mobility was not found.

Calibration, Randomization and Allocation Concealment

Initially, a total of 3 non-study subjects presenting generalized AgP were selected. The designated examiner (RCVC) measured the relative clinical attachment level (RCAL), i.e., the distance from the bottom of the pocket to the stent margin and PPD in all patients, twice, within 24 hours, with an interval of at least 1 hour between the examinations. The intra-class correlation was calculated for each parameter, resulting in 91% reproducibility for RCAL and 94% for PPD. This calibration was repeated annually.

Family study—Initially, a total of 72 generalized AgP patients and their relatives were recruited. However, several families were excluded during the course of the study, due to varied reasons including lack of further interest in the study, no contact with relatives, family living in another state, adopted probands, and the fact that some patients simply did not wish to have a dental examination performed. The family study was then accomplished with thirty nine individuals, i.e., thirteen subjects affected by generalized AgP (probands) and their parents, as described below:

1. *AgP probands (n = 13)*: Subjects presented periodontal pockets with clinical attachment loss and radiographic bone loss in at least 3 teeth (excluding 1st molars and incisors), at least eight teeth with a probing pocket depth (PPD) > 5mm and

bleeding following pocket probing. At least two of the eight qualifying teeth must have PPD > 7 mm; age inferior than 35 years.

2. *AgP parents (n = 8)*: The progenitors presented the same periodontal characteristics as AgP probands, but had experienced multiple tooth loss due to the progress of the disease; in addition, they were older than 35 years.
3. *Non-AgP parents (n = 18)*: subjects did not show any sites with clinical attachment loss and probing pocket depth ≥ 3 mm and presented full-mouth bleeding score (FMBS) < 25%; tooth mobility was not found.

With respect to the progenitors, the diagnosis of AgP was obtained after careful intra-oral examination and evaluation of medical and dental records, with particular regard to periodontal treatment. When patients had experienced multiple tooth loss, efforts were made to understand the cause of the tooth loss and to obtain past radiographic and dental data. In some cases, contact was made with their respective private dentists.

Case-control study—A total of 108 control subjects and 76 generalized AgP patients were included in this case-control study:

1. *Control group (n = 108)*: Subjects did not show any sites with clinical attachment loss and probing pocket depth ≥ 3 mm and presented full-mouth bleeding score (FMBS) < 25%; tooth mobility was not found.
2. *Generalized AgP group (n = 76)*: Subjects presented periodontal pockets with a clinical attachment loss and radiographic bone loss in at least 3 teeth (excluding 1st molars and incisors); at least eight teeth with a probing pocket depth (PPD) of > 5 mm and bleeding following pocket probing. At least two of the eight qualifying teeth must have PPD > 7 mm; age inferior than 35 years.

Genetic analysis

Isolation of genomic DNA—The sampling of buccal epithelial cells was performed as described before.²² DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation, dissolved in TE buffer [10mM Tris (pH 7.8), 1mM EDTA] and its concentration estimated by measuring absorbance at 260 nm using a spectrophotometer (Thermo Fisher Scientific, Evolution 60, Madison WI, USA).

PCR—A 349 bp fragment of the *IL8* gene promoter (GenBank M28130) was PCR-amplified with the primer set: (forward) 5'-CATGATAGCATCTGTAATTAAGTG 3' and (reverse) 5'-CTCATCTTTTCATTATGTCAGAG- 3'. PCR was carried out in a total volume of 10 μ l, containing 75ng of genomic DNA, 0.1 μ M each primer and 1U of Go *Taq* Green Master Mix (Promega Corporation, Madison, WI, USA). The solution was incubated for 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 51°C and 1min at 72°C, with a final extension of 72°C for 7 min.

Genotyping IL8 SNP rs4073—The restriction endonuclease digestion was prepared using a 5 μ l aliquot of *IL8* PCR products mixed with a 5 μ l solution containing 1 μ l 10 X NE Buffer (50mM NaCl, 10mM Tris-HCl, 10mM MgCl₂, 1mM dithiothreitol, pH 7.9), 0.25 μ l

MfeI (10,000 U/ml) (New England Biolabs, Ipswich, MA, USA) and 3.75 μ l sterile deionized H₂O. The solution was incubated at 37°C overnight. The A allele completes a corresponding restriction endonuclease site, while the T allele does not. The length difference of the RFLP fragments was 147bp and 55bp (TT 349bp; TA 349bp, 202bp and 147bp; AA 202bp and 147bp). Aliquots of 5 μ l of the RFLP products were electrophoresed on 10% vertical non-denaturing polyacrylamide gel at 20 mA. The gel was silver stained by a nucleic acid gel stain (SYBR Gold, Eugene, Oregon, USA). All samples were genotyped in duplicate and the genotyping was performed blind with respect to clinical diagnosis.

Statistical analysis

Study Power calculations—We will fit several models in our case-control population study, controlling the false positive rate with an FDR (false discovery rate) approach. While it is difficult to calculate precise statistical power given this combination of methods, we use the following calculation to justify that our proposed sample size is capable to detect genes of interest. The statistical power of the sample was calculated using the Purcell calculator,²³ assuming a disease prevalence of 5.5%,²⁴ significance level of 0.05 and r^2 of 0.8 between the tested SNP and the causal locus for sample sizes of 76 cases and 108 controls. Note that given the r^2 value, the power depends only on the risk allele frequency, not on the marker allele frequency.

Transmission disequilibrium test—The over transmission of the A allele in the families with generalized AgP was analyzed by the transmission disequilibrium test (TDT - Family-Based Association Test package, FBAT - 2.0.3, Harvard University, Boston, MA, USA).²⁵ The program constructs, by default, a test of the null hypothesis: no linkage and no association.²⁶ The TDT test analyses whether the transmission of an allele from parents who are heterozygous at the marker tested to the probands deviates significantly from the expected value of 50%.

Chi-square test and multivariate logistic regression—Association analysis between *IL8* SNP rs4073 and generalized AgP in cases and controls was performed using chi-square test and multivariate logistic regression modeling adjusted for gender, age and ethnicity (R Development Core Team, Vienna, Austria) using the glm() function. There were three ethnic groups enrolled in this study: Caucasoid, mestizo and african-american; the statistic program automatically dropped the African-american individuals as reference to the regression analyses. Similarly, the statistic program dropped the TT genotype as reference to the regression analyses, as can be observed in the *p* value and coefficient columns (table 4). Analysis of Hardy-Weinberg equilibrium was used to test deviation of genotype distribution (BioEstat ver.5.0, Belém, PA, Brasil).

Results

Table 1 shows the results of the study power calculations and the size of the study population was lower than necessary for giving results with 80% of statistical power, with an alpha error of 10%. Table 2 shows the demographic and clinical characteristics of the families and unrelated individuals.

Family study

The results showed that all 13 families were informative for TDT analysis, having at least one parent who was heterozygous for *IL8* rs4073. There was no evidence of deviation from Hardy-Weinberg equilibrium ($p = 0.2357$). Table 3 shows that there was a balanced transmission between the alleles (allele T $Z = 1.213$, $p = 0.2252$; allele A $Z = -1.213$) and lack of association with generalized AgP ($p = 0.2252$). In addition, there was balanced distribution of alleles frequencies as well (allele T = 52% and allele A = 48%) and individuals with the T allele are approximately 1.1 times to develop periodontitis (Odds ratio (OR) = 1.1; 95% Confidence Interval (CI) = 0.45 – 2.7).

When considering all affected family members (AgP probands – $n = 13$ plus AgP parents – $n = 8$, the heterozygous genotype (TA) was the most frequent (66.7%), followed by the TT genotype (19%) and by the AA genotype (14.3%). We also found that 80% of the affected family members presented at least one A allele.

Case-control study

There was no evidence of deviation from Hardy-Weinberg equilibrium ($p > 0.05$). Multivariate analysis showed a significant and inverse association between age and generalized AgP ($p = 0.000$; coefficient -0.07); on the other hand, lack of association between genotypes, ethnic groups and generalized AgP was observed (table 4). The genotypes frequencies in the control group were TT = 35.2%, TA = 52.8% and AA = 12.0%, resulting in a frequency of 38% of the risk allele A, and carrier frequency of at least one A allele of 64.8%. On the other hand, the frequencies of TT, TA and AA genotypes in the generalized AgP group were 19.7%, 65.8% and 14.5%, respectively, resulting in an A allele frequency of 47% and carrier frequency of at least one A allele of 80.3%. Individuals with the TA genotype are approximately 1.73 times to develop periodontitis (Odds ratio (OR) = 1.73; 95% Confidence Interval (CI) = 0.94 – 3.15) and with the A allele are approximately 1.44 times to develop periodontitis (Odds ratio (OR) = 1.44; 95% Confidence Interval (CI) = 0.94 – 2.19) (Table 4).

Discussion

In the present study we investigated the transmission distortion and association patterns of *IL8* rs4073 using family-based and case-control study designs. The *IL8* rs4073 is transmitted at linkage disequilibrium with 5 other alleles in the coding sequence of the *IL8* gene, including the functional +781 T allele that influences *IL8* gene transcription levels.²⁷ Hacking et al.²⁷ showed that the *IL8* rs4073, located at 251 base pairs from the transcription start site in the *IL8* gene promoter, did not show functional activity in response to TNF stimulation. However, the authors reported that this variant is in linkage disequilibrium with a functional variant in the *IL8* gene, composing combined genotypes that may be of further functional relevance. Moreover, the authors report the occurrence of two major haplotypes: Haplotype 1 (52%): T_{allele -251}/T_{allele +396}/C_{allele +781}/insA_{allele +1238}/C_{allele +1633}/A_{allele +2767} and Haplotype 2 (41%): A_{allele -251}/G_{allele +396}/T_{allele +781}/delA_{allele +1238}/T_{allele +1633}/T_{allele +2767}. The haplotype-2, which contains the -251A allele, up regulates the

IL8 transcription and may lead to increased expression in individuals heterozygous for haplotypes -1 and -2.

Our family-based analyses did not detect evidence of over transmission of *IL8* rs4073 alleles in families with AgP. However, we observed a relatively high risk for relatives of AgP patients to also have AgP (30.8%, data not shown), which is higher than what has been previously reported (10%).⁴ Notwithstanding the prevalence of AgP has been reported to vary greatly between populations and generally, low prevalence rates (~0.1% to 0.5%) have been found among Caucasians in developed countries.²⁸ Further, the prevalence of AgP in Brazil has been studied^{24,29–31} and the authors founded prevalence rates that may range from ~1.1% to 5.5%. Discrepancies among the results of the present and the aforementioned studies may be related to our limited sample size for families with AgP, which might account for the lack of association with *IL8*. One may argue that by selecting only non-smoking individuals for the study the ability of achieving large sample size is limited.

The advantage of a family study design, compared with a case-control design, is the elimination of control subjects whose genetic background differs systematically from those of cases;³² on the other hand, a limitation of family based analysis is that it does not provide a direct measure of relative risk for disease within the population as a whole. It has been proposed that this may be overcome by using the alleles that are not transmitted from heterozygous parents to affected offspring to estimate allele frequencies within the general population.¹⁵

We also investigated the association of *IL8*rs4073 in a case-control dataset composed of individuals affected by generalized AgP and unaffected unrelated controls. We found an increased frequency (65.8%) of the heterozygous genotype (TA) in subjects with generalized AgP, when compared to controls and although this could be of interest, it was not statistically significant. This percentage is higher than reported for African-American (37%) and European populations (~40%–46%), although our findings are in agreement with the percentage of the A allele found in the Europeans (40%).³³

A concern with case-control studies is that cases and controls must be properly matched especially for ethnicity, to overcome any possible effects of population stratification acting as potential confounders in the analyses. Since the Brazilian population is known to present some degree of admixture, we used multivariate regression analysis adjusted for gender, age and ethnicities, which are important factors known to influence the pathogenesis of AgP. The results showed a lack of association of gender and generalized AgP, as well as for ethnicities. However, the analysis of the case-control data set demonstrated a significant and inverse association between age and generalized AgP, demonstrated by an effect size of -0.07 (negative coefficient). This inverse association is in agreement with the peculiarity of early occurrence of AgP, i.e., the older the individual, the lower the probability for him to become an AgP patient.

A few studies have focused on the association of the SNP 4073 in the *IL8* gene with chronic periodontitis.^{34–36} A recent study with the same *IL8* SNP (rs4073), also in a Brazilian population,³⁴ reported lack of association with chronic periodontitis. In a subsequent

study,³⁵ these same authors investigated the SNP (rs4073) as part of combined haplotypes (–845TC, –738TA and –353AT), and reported that nonsmokers individuals carrying the TAT/CTA genotype were six times more likely to develop chronic periodontitis. Interestingly, the current study also reported lack of association with aggressive periodontitis. In this context, the investigation of the SNP (rs4073) as part of haplotypes might introduce some new perspectives in future studies.

In another study of the association between the *IL8* rs4073 and generalized chronic periodontitis,³⁶ the TA genotype was associated with increased risk, at a frequency of 52.8% in controls versus 74.6% in chronic periodontitis patients. In the current study, the authors found a frequency of 65.8% of the TA genotype in the generalized AgP patients. Therefore, a lower frequency of the TA genotype was found in the AgP patients, compared to the chronic patients. This fact leads us to think about on the possible differences between the genetic profiles of chronic and aggressive periodontitis. In addition, Andia et al.³⁶ analyzed *IL8* mRNA transcripts in gingival biopsies and found substantial variability in expression levels, however, markedly increased levels were detected in individuals with chronic periodontitis presenting the TA genotype. It would be nice to investigate the correlation between the allele frequencies with *IL8* mRNA transcripts levels also in the AgP patients; however, the authors did not have access to gingival biopsies of the individuals in this study.

The present study has an insufficient statistical power. Statistical power may depend on a number of factors, including the sample size used to detect the effect. As it was mentioned before, we had some difficulties to achieve the perfect sample size; therefore, there is a probability of a false negative outcome and we have to be very careful to state that there is no association between the SNP (rs4073) and the population studied.

Given our incomplete knowledge of the pathogenesis of periodontal diseases, the search for specific genetic and microbiologic profiles that may be associated with AgP has been crucial. After the investigation, we have concluded that, in the studied population, the SNP (rs4073) was not associated with generalized AgP in unrelated individuals; moreover, we founded a lack of over transmission of the alleles in families with AgP, from Brazilian individuals. Further, our findings highlight the importance of usual examination in the offspring of patients with AgP, targeting preventive therapies to reduce possible damages associated with the disease, such as pronounced tooth loss. Future investigations should include larger sample sizes, haplotypes studies and additional populations to confirm the present results.

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Table 1

Study power calculation results.

Case-control statistics: allelic 1 degree of freedom (df) test using a dominant versus recessive allele (B versus b) condition

Sample NCP (sample noncentrality parameter) = 5.459

Alpha	Power	N cases for 80% power
0.1	0.7555	86
0.05	0.6468	109
0.01	0.4054	162
0.001	0.17	237

Table 2

Demographic and clinical characteristics of the families and unrelated individuals.

Characteristic	Family-Based			
	Unaffected (n=18)	Affected (n=21) probands (n=13) parents (n=8)	Controls (n=108)	Case-Control AgP (n=76)
Age (years)				
Mean ($\pm$ SD)	52.1 $\pm$ 7.0	26.5 $\pm$ 4.6 $\pm$ 8.27	37.2 $\pm$ 12.8	29.2 $\pm$ 5.6
Gender (%)				
Female	50.0	51.6	66.3	80.3
Male	50.0	48.4	33.7	19.7
Ethnic group (%)				
Caucasoid	55.5	57.1	75.0	59.0
Mestizo	38.9	23.9	10.2	20.5
Afro-American	05.6	19.0	12.9	20.5
PPD(omm) (full mouth mean$\pm$SD)	2.4 $\pm$ 1.2	6.6 $\pm$ 0.5	2.0 $\pm$ 0.7	7.1 $\pm$ 1.3
CAL (full mouth mean$\pm$SD)	2.9 $\pm$ 0.8	7.8 $\pm$ 1.1	2.2 $\pm$ 1.2	7.5 $\pm$ 1.9
PI	23.3 $\pm$ 15.0	41.9 $\pm$ 23.4	25.4 $\pm$ 4.6	42.7 $\pm$ 24.9
GI	12.1 $\pm$ 2.8	37.7 $\pm$ 18.0	11.3 $\pm$ 2.2	35.6 $\pm$ 17.8

Notes: PPD, periodontal probing depth; CAL, clinical attachment level; PI, plaque index; GI, gingival index.

Table 3

Genotypes and alleles distribution of the *IL8* SNP (rs4073) in unaffected and affected family members with AgP, and results of transmission disequilibrium analysis.

Genotype	Family dataset		Allele	Frequency	Number of informative families	P value	Odds ratio	95% CI
	Unaffected	Affected						
AA	5(19.2%)	1(7.7%)	T	0.52	13	0.2252	1.1	0.45
TA	17(65.4%)	9(69.3%)	A	0.48	13			
TT	4(15.4%)	3(23%)						

Family data set of unaffected and affected individuals was given as *n* (%). *P* value obtained by the transmission disequilibrium test (TDT). CI indicates confidence interval.

Case-control data set: Genotype, age and ethnic group results adjusted by multivariate logistic regression modeling and alleles distribution of the *IL8* SNP (rs4073).

Table 4

SNP (rs4073)	Control (N=108)	Generalized AgP	Coefficient 95%CI	P value	Odds
<i>Genotype</i>					
AA	13 (12.0%)	11 (14.5%)	0.08	0.88	
TA	57 (52.8%)	50 (65.8%)	0.18	0.60	1.73 0.94–3.15
TT	38 (35.2%)	15 (19.7%)	reference	reference	
<i>Alleles</i>					
A	83 (38%)	72 (47%)		2.19	1.44 0.94–
T	133 (62%)	80 (53%)			
<i>Age</i>					
			–0.07	0.00	
<i>Gender</i>					
			–0.48	0.20	
<i>Ethnic group</i>					
Caucasoid			–0.43	0.29	
Mestizo			0.20	0.70	
African-American			reference	reference	

SNP indicates single nucleotide polymorphism; AgP indicates generalized aggressive periodontitis; N indicates number of informative individuals. Coefficient and *p* value were obtained by multivariate logistic regression modeling.