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Polymorphism in the tumor necrosis factor-alpha gene (TNFA -308 G/A) is not associated with susceptibility to chronic periodontitis in a Brazilian population

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Abstract

Objective: Tumor necrosis factor-alpha (TNF-α) is a major mediator of the immune-inflammatory response and may play an important role in the pathogenesis and progression of chronic periodontitis. Polymorphisms in the promoter of the TNFA gene have been associated with some types of inflammatory diseases. The present study investigated the association between a single-nucleotide polymorphism (SNP) of the TNFA (G-308A) gene and chronic periodontitis in Brazilians. **Methods:** One hundred and thirteen (113) over 25 years were divided according to the severity level of periodontal disease: 44 healthy individuals (control group), 31 subjects with moderate and 38 patients with severe periodontitis. Genomic DNA was obtained from epithelial cells. The samples were analyzed for TNFA (G-308A) polymorphism using polymerase chain reaction-restriction fragment length polymorphism techniques. The significance of the differences in the genotype frequencies of the polymorphism was assessed by Chi-square test ($p<0.05$). **Results:** No significant differences in the genotype distribution and allele frequency were found between control and groups with periodontitis. **Conclusion:** It was concluded that TNFA (-308) polymorphism was not associated with chronic periodontitis. Other polymorphisms in this or/and other genes of the host inflammatory response might be involved in determining susceptibility to periodontitis in the study population.

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Introduction

Bacterial lipopolysaccharides (LPS) activate monocytes of inflamed periodontal tissues to produce cytokines. Tumor necrosis factor-alpha (TNF-α) is an important immune mediator and a pluripotent proinflammatory cytokine that is able to induce bone resorption [1].

TNFA gene is encoded on chromosome 6 inside the HLA region [2] and its biological activities have raised the possibility that polymorphisms within this gene might contribute to genetic association to diverse immune-inflammatory diseases. In fact, polymorphisms in TNFA gene have been implicated in the pathogenesis of a large number of human diseases. Within the promoter region of TNFA, a biallelic polymorphic site at position -308 has been reported to influence the production of TNF-α protein [3]. Allele A of TNFA (-308) polymorphism has been positively associated with asthma [4] and ulcerative colitis [5].

Vol 3, No 1 (2015) DOI 10.5195/d3000.2015.27

Table 1. Baseline clinical parameters of the subject population (n=113).

	Healthy (n=44)	Moderate (n=31)	Severe (n=38)
Age (years) Mean (± SD)	43.2 (±14.0)	36.9 (±11.2)	43.6 (±14.4)
Gender %			
Female	68.2	80.6	84.2
Male	31.8	19.4	15.8
Ethnic Group %			
Caucasian	84.1	77.4	68.4
Afro-American	6.8	16.1	13.2
Mulatto	6.8	6.5	18.4
Japanese	2.3	0.0	0.0

Epidemiological studies indicate that chronic periodontitis (CP) is widespread among the Brazilian population [6, 7]. TNFA mRNA was significantly more frequent in diseased than in healthy gingival biopsies [8], suggesting a role of this cytokine in the development of CP. Increased levels of TNF-α in periodontal tissues were associated with genotypes carrying allele A [9]. Because of the increasing evidence that genetic variations within the TNFA locus are important in determining susceptibility to periodontitis, this study aimed to investigate the possible link between the polymorphism at position -308 (G→A) of TNFA gene promoter and chronic periodontitis in a Brazilian population.

Materials and Methods

Subject Selection

A convenience sample of 113 unrelated, non-smoking subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba - UNICAMP. The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in table 1.

All subjects were in good general health and had at least 20 teeth in the mouth. Subjects were not included if presented: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis or current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study (approved by the Ethical Committee in Research at FOP/UNICAMP, protocol 63/99).

Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of clinical attachment loss (CAL), tooth mobility, gingival recession and observation of bleeding on probing, according to Lindhe et al. [10]. Measurements of probing depth and attachment level were recorded at 6 points around each tooth. Subjects were included in clinical categories according to:

CP severity:
1) Healthy group: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 44) in at least 3 teeth in at least 2 different quadrants;
2) Moderate Periodontitis: Patients with tooth sites exhibiting ≥3 mm and <7 mm CAL (n = 31) in at least 3 teeth in at least 2 different quadrants;
3) Severe Periodontitis: Patients with teeth exhibiting ≥7 mm CAL (n = 38) in at least 3 teeth in at least 2 different quadrants.

Sampling

The sampling of epithelial buccal cells was performed as described by Trevilatto and Line [11]. Briefly, 113 individuals undertook a mouthwash after 1 min, containing 5 ml 3% glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shaken into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 µl of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% SDS]. The samples were then frozen at -20oC until used for DNA extraction.

DNA Extraction

Vol 3, No 1 (2015) DOI 10.5195/d3000.2015.27

After defrosting, samples were incubated overnight (ON) with 100 ng/ml proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 µl TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Table 2. Distribution of the TNF-α genotypes in the healthy group and in groups with moderate and severe chronic periodontitis.

Group	GG	GA	AA	p value
Healthy	37 (84.0)	7 (16.0)	0 (0.0)	0.289
Moderate	24 (77.4)	7 (22.5)	0 (0.0)	
Severe	31 (81.6)	0 (0.0)	2 (5.3)	

Polymerase Chain Reaction (PCR) and Restriction Fragment Polymorphism (RFLP)

Polymorphism in the TNF-α gene at position -308 (rs1800629) The oligonucleotides 5'-AGGCAATAGGTTTTGAGGGCCAT-3' and 5'-TCCTCCCTGCTCCGATTCCG-3'. Amplification reactions were performed with 500 ng genomic DNA in a total volume of 50 µl, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 µM of each primer, 200 µM each dATP, dCTP, dGTP and dTTP, and 2.5 units Taq DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). Cycling was 2 cycles of 3 min at 94°C, 1 min at 60°C and 1 min at 72°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, with a final extension of 7 min at 72°C. The products were digested with 3 U per 25 µL reaction of NcoI at 37°C ON to detect allele 1 [G] (20 bp + 87 bp) and allele 2 [A] (107 bp).

Electrophoresis gel

Restriction products were visualized by electrophoresis on vertical 10% non-denaturing polyacrylamide gels in 1X TBE (89 mM Tris-Borate, 89 mM boric acid, 2 mM EDTA), followed by silver staining (Bio-Rad Silver Stain Kit).

Statistical analysis

The allele ratio and genotype distribution of periodontitis patients and healthy control subjects were analyzed with Chi-square test. A p-value <0.05 was considered significant.

Results

There was no significant difference in the genotype distribution (p=0.289) between healthy control and periodontitis patients in the polymorphism TNFA (-308), neither was observed statistical variation in the frequency of the alleles (p=0.674). The distribution of the genotype and the frequencies of the alleles for the polymorphism studied are shown in tables 2 and 3, respectively. Allele 2 was carried by 19.5% (22/113) of the subjects, with 1.8% (2/113) of homozygous.

The allele distribution in the study population was consistent with Hardy-Weinberg equilibrium. The moderate and severe groups (table 1) were combined and compared to the healthy group in order to seek a better biological explanation of the results. The results of analyzes of the additive model (p = 0.503), a dominant model for the L (p = 0.559) and for the recessive allele G (p = 0.255) allele were not significant.

Discussion

Because of their frequent presence in diseased sites, it has been postulated that cytokines such as interleukin-1 (IL-1) and TNF-α may be indicators of the active phase of periodontal disease [12]. Tumor necrosis factor-alpha, a pro-inflammatory cytokine, has been detected in gingival crevicular fluid and gingival tissues from individuals with periodontitis [13]. This cytokine is a potent immunologic mediator that, in addition to its inflammatory effects, increases bone resorption and regulates fibroblast proliferation [14].

A number of single nucleotide polymorphisms (SNPs) has been identified in the TNFA promoter and associated with certain chronic inflammatory diseases, including cerebral malaria [15], multiple sclerosis [16], ulcerative colitis [17], Alzheimer's disease [18], chronic bronchitis [19] and rheumatoid arthritis [20]. In cerebral malaria TNF-α is of major importance for the pathology [15] and individuals homozygous for the TNFA (-308) A allele carry a 7-fold higher risk of death or severe neurological sequelae due to cerebral malaria.

In this study, no association of TNFA (-308) polymorphism with CP was found. However, in another Brazilian population, Trombone et al. [9] found a frequency (17.7%) of the TNFA (-308) A allele in the CP group. Kornman et al. [21] found an allele A carriage rate of 28.5% at

Vol 3, No 1 (2015) DOI 10.5195/d3000.2015.27

Table 3. Distribution of the TNF-α alleles in healthy, moderate and severe groups with chronic periodontitis.

Allele	Healthy n (%)	Moderate n (%)	Severe n (%)	p value
G	81 (92.0)	55 (88.7)	67 (88.0)	0.674
A	7 (8.0)	7 (11.3)	9 (12.0)	

TNFA (-308) locus in North-American Caucasians, but no association with CP. We found an allele A carriage rate of 20.7% (18/87) for our Caucasians.

Although the study sample was mostly composed by Caucasians, the Brazilian white population is heterogeneous. Recent article has not suggested grouping Brazilians into ethnic groups based on color, race and geographical origin because Brazilian individuals classified as white or black have significantly overlapping genotypes, probably due to miscegenation [22]. Reporting the Caucasoid population, there is a predominance of Italian, Spanish, and Portuguese heritage. In a meta-analysis by Nikolopoulos et al. [23] the great majority of studies were carried out in Caucasian populations and no association of TNFA (-308) was found neither with aggressive nor chronic periodontal diseases. A lack of association of this polymorphism in patients with early-onset periodontitis was also found in a Japanese population [24]. However, Lin et al. [25] observed that allele A may increase the odds of having moderate-to-advanced aggressive periodontitis. Galbraith et al. [26] reported the frequency of TNFA (-308) allele G significantly greater in patients with advanced periodontitis. Recently, a new meta-analysis study was performed [27] and stratification by ethnicity showed that the A allele was associated with periodontitis in Brazilians, in spite of the lack of association showed in the studies included in the meta-analysis for CP [28-30].

There has been an evidence that allele A is over-represented in diseases where TNF-α levels are associated with poor prognosis [31]. Allele A of TNFA (-308) polymorphism seems to be associated to increase in the production of TNF-α cytokine [9]. Although the role TNF-α plays in leading to tissue destruction, it is worth mentioning the need for evaluation of other potential candidate genes as contributors to periodontitis, since chronic periodontitis may possibly represent a polygenic disease.

Analysis in some of other study cytokine genes (IL4, MMP1, VDR, IL1) revealed positive associations with chronic periodontitis in the same Brazilian patients [32-35]. Those studies may provide pieces of information with regard to which factors could indeed be implicated in the process of disease in this population. In spite of the reduced number of individuals in each group, it seems that this sample was composed of well characterized phenotypes, once positive associations could be identified for some gene polymorphisms in previous studies [33, 35, 36]. Besides, only a small functional part of the gene was investigated, which does not mean that this gene could not be involved in the susceptibility control of chronic periodontitis. Thus, an investigation of tag SNPs, which physically represent the gene as a whole, should be recommended.

It was concluded that TNFA (-308) polymorphism was not associated with chronic periodontitis in the population studied.

Acknowledgements

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Vol 3, No 1 (2015) DOI 10.5195/d3000.2015.27

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Vol 3, No 1 (2015) DOI 10.5195/d3000.2015.27

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