Lack of association between the TNF-α -308 (G/A) genetic polymorphism and periodontal disease in Brazilians

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Abstract: This study evaluated the frequency of the tumor necrosis factor-alpha (TNF-α) -308 G/A polymorphism in Brazilians with periodontal health (PH = 51), chronic periodontitis (CP = 74) and generalized aggressive periodontitis (GAgP = 38). Human DNA was obtained from mouthwash samples and TNF-α genotyping was performed by PCR and RFLP analyses. Differences in clinical and genetic parameters among groups were sought by Kruskal-Wallis, χ^2 and Fisher's exact tests. The allele -308G was detected in 91.7%, whereas the allele -308A was found in 35.4% of all subjects. No significant differences were observed in the frequency of these alleles ($\chi^2 = 2.610$, $p > 0.05$) and the genotypes G/G, G/A, and A/A ($\chi^2 = 2.547$, $p = 0.636$) among groups. The data suggest that the TNF-α -308 G/A polymorphism is not associated with periodontitis in this Brazilian population.

Descriptors: TNF-alpha; Periodontal diseases; Genetic polymorphism; PCR.

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Introduction

The cytokine TNF-α has been found at high levels in gingival crevicular fluid and gingival tissues from periodontitis lesions.[1] TNF-α was clearly identified as a potent inducer of tissue destruction and bone resorption in different forms of periodontal disease.[2] A guanine (G) to adenine (A) transition at position -308 of the TNF-α promoter affects a consensus sequence for a binding site of the transcription factor AP-2.[3] Carriage of the rare -308 A allele is associated with significantly greater TNF-α production and transcription.[3] In addition, the A allele has been associated with increased risk for various non-related infectious and inflammatory diseases,[4] including periodontitis.[5,6] In contrast, other investigators have failed to corroborate the association between this polymorphism and increased risk for periodontal diseases.[7-11] The frequency of genetic polymorphisms may vary considerably among distinct ethnic groups, so that the application of such markers for diagnosis and prognosis of periodontitis should be examined in different populations.[12] This study determined the frequency of the -308 (G/A) TNF-α alleles and genotypes in individuals with different periodontal *status* from a Brazilian population.

Material and Methods

Human subjects and clinical assessments

A total of 163 adult subjects who sought dental treatment at the Dental School of the Federal University of Rio de Janeiro were recruited for the study. Informed consent was obtained from all individuals. The study protocol was approved by the Review Committee for Human Subjects of the Clementino Fraga Filho University Hospital. Exclusion criteria included pregnancy, use of local or systemic antimicrobials in the previous 6 months, diabetes, and other systemic conditions that could affect the periodontal *status*. All subjects had at least 10 teeth and were over 18 years of age. Clinical measurements were performed at 6 sites/tooth and included probing depth (PD), clinical attachment level (CAL), presence or absence of supragingival biofilm (SB), bleeding on probing (BOP) and suppuration (SUP). Subjects were categorized into: periodontal health controls (PH = 51; no sites with PD and/or CAL > 3 mm and no more than 10% of sites with BOP); chronic periodontitis (CP = 74; at least 10% of teeth with PD and/or CAL ≥ 5 mm, or 20% of teeth with PD and/or CAL ≥ 4 mm); and generalized aggressive periodontitis (GAgP = 38; ≥ 30% of teeth with PD and/or CAL ≥ 5 mm, or ≥ 60% of teeth with PD and/or CAL ≥ 4 mm). Patients were defined (by self-reporting) as White, African-Brazilian, or others. Individuals were classified as smokers, never-smokers and former smokers (subjects who had stopped smoking for at least 2 years).

Determination of -308 (G/A) TNF-α genotypes

Mouthwash samples were obtained from patients as previously described.[13] Human genomic DNA was isolated from samples by using the QIAmp DNA Mini Kit (QIAGEN, Valencia, CA, USA), and stored at –20°C. PCR was performed in a 50 μL reaction mix containing 100 ng of DNA, 5 μL of 10 X PCR buffer (100 mM TrisHCl, pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 U of *Taq* polymerase (PROMEGA®, Madison, WI, USA), and 0.2 pM of each primer[14] (sense primer 5' -AGG CAA TAG GTT TTG AGG GCC AT- 3' and antisense primer 5' -TCC TCC CTG CTC CGA TTC CG- 3'; Bio-Synthesis®, Inc., Lewisville, TX, USA). The PCR program included a step of 95°C for 10 min, followed by 38 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min. Subsequently, 20 μL of the amplicon (107 bp fragment) were digested with 5 U of *Nco I* (PROMEGA®) at 37°C overnight and the generated DNA fragments were analysed by electrophoresis on a 2.5% agarose gel.

Statistical analysis

Analyses were performed using the software SPSS (SPSS Inc® v.13 Chicago, IL, USA). Clinical measurements were computed for each subject and averaged across subjects within the groups. Differences on clinical parameters among groups were sought using Kruskal-Wallis, Mann-Whitney (between pairs of groups) and χ^2 tests. The frequency of alleles and genotypes were compared among groups by χ^2 and Fisher's exact tests, and odds ratios with 95% confi-

dence intervals were determined. In order to control for age and race, the data were stratified by these categories and analyzed by Kruskal-Wallis and χ^2 tests. A 5% significance level was set for all analyses.

Results

Demographic and clinical features

The demographic data of the 3 clinical groups are summarized in Table 1. PH and GAgP patients were significantly younger than CP individuals ($p < 0.01$). A greater % of White patients was observed in the PH group (81%), whereas the CP and GAgP groups presented a higher proportion of African-Brazilians (22% and 14%, respectively) and individuals of mixed racial background (19% and 50%, respectively) ($p = 0.002$). In general, most of the patients were never-smokers (78.5%). Nevertheless, a higher frequency of current and former smokers with chronic periodontitis was detected ($p < 0.001$).

Table 2 shows that statistically significant differences among groups were observed for all clinical parameters ($p < 0.01$). When pairs of groups were compared, no significant differences were found for number of missing teeth, BOP, SB, SUP, and CAL > 6 mm between the CP and GAgP groups. In order to control for smoking and race, comparisons of clinical parameters among groups were carried out only in never-smokers and White individuals. Even though, differences among groups were statistically significant for all clinical measurements ($p < 0.001$; data not shown).

Table 1 - Demographic parameters (mean ± SD) of Periodontally Healthy (PH), Chronic Periodontitis (CP), and Generalyzed Aggressive Periodontitis (GAgP) subjects of the study population.

Parameters		PH (N = 51)	CP (N = 74)	GAgP (N = 38)
Age *‡		30 ± 10	48 ± 12	31 ± 6
(%) Males		31	41	18
Race (%)**	Whites	81	59	36
	African-Brazilians	8	22	14
	Others	11	19	50
Smoking (%)**	Never-smokers	88	61	100
	Former-smokers	8	27	0
	Current-smokers	4	12	0

* Refers to $p < 0.01$, Kruskal-Wallis test, and ‡, to Mann-Whitney test; ** refers to $p < 0.01$, χ^2 test.

Frequency of the TNF-α (-308 G/A) alleles and genotypes

The allele distributions of the TNF-α among clinical groups are shown in Table 3. No differences were found between observed and expected distributions of genotypes for the PH control group. Thus, the allele distribution was assumed to be in Hardy-Weinberg equilibrium. A high incidence of allele G was observed in all groups. Likewise, the % of subjects carrying at least one copy of the G allele was greater than subjects carrying allele A. However, no significant differences in the distribution of these alleles were found among groups ($\chi^2 = 2.610$, $p > 0.05$ for allele frequency; $\chi^2 = 1.01$, $p = 0.604$ for G+ and $\chi^2 = 2.12$, $p = 0.345$ for A+ individuals). When PH subjects were compared with the CP or GAgP patients, no significant differences between groups were observed for allele frequency. Also, no significant differences in the distribution of these alleles

Table 2 - Full-mouth clinical parameters (mean ± SD) of Periodontally Healthy (PH), Chronic Periodontitis (CP), and Generalyzed Aggressive Periodontitis (GAgP) subjects of the study population.

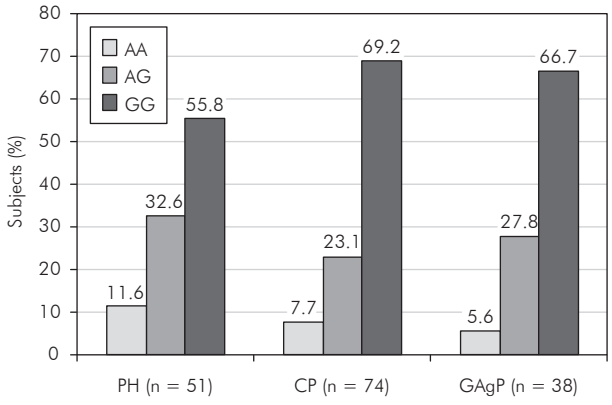
Parameters		PH (N = 51)	CP (N = 74)	GAgP (N = 38)
N of missing teeth *†		2.3 ± 3.6	5.5 ± 4.8	4.4 ± 4.7
PD (mm) *†‡		1.7 ± 0.3	2.7 ± 0.9	3.3 ± 0.9
CAL (mm)*†		1.8 ± 0.3	3.6 ± 1.5	3.8 ± 1.1
% sites with:	BOP *†	4.3 ± 4.3	41.2 ±26.6	48.6 ±28.4
	SB *†	13.4 ±16	46.3 ±30	37.4 ±29
	SUP *†	0	0.6 ± 1.6	2.3 ± 4.9
	PD ≤ 4 mm *†‡	100	88 ±16	75 ±20.3
	PD 5-6 mm*†‡	0	8.5 ± 9.4	19 ±14
	PD > 6 mm*†‡	0	3.5 ± 8.5	6 ± 8.5
	CAL ≤ 4 mm*†‡	100	73 ±27	66 ±24
	CAL 5-6 mm*†‡	0	16 ±15	24 ±14
	CAL > 6 mm*†	0	11 ±17	10 ±14

* Refers to $p < 0.01$ (Kruskal-Wallis test); † refers to $p < 0.01$ (Mann-Whitney between PH and CP, and PH and GAgP); ‡ refers to $p < 0.01$ (Mann-Whitney between CP and GAgP); PD = pocket depth; CAL = clinical attachment level; BOP = bleeding on probing; SB = supragingival biofilm; SUP = suppuration.

Table 3 - Frequency of alleles in the TNF-α -308 G/A gene polymorphism in Periodontally Healthy (PH), Chronic Periodontitis (CP), and Generalyzed Aggressive Periodontitis (GAgP) subjects of the study population.

		PH	CP	GAgP	PH *vs.* CP ***	PH *vs.* GAgP ***
		n = 51	n = 74	n = 38	OR (95%CI)	OR (95%CI)
Allele frequency * †	TNF-α -308G	72.5%	81%	81.6%	0.616 (0.34-1.12)	0.596 (0.29-1.23)
	TNF-α -308A	27.5%	19%	18.4%		
Polymorphic allele positivity **	G+ §	88.4%	92.3%	94.4%	1.5 (0.43-5.82)	4.5 (0.40-12.3)
	A+ ⊥	44.2%	31%	33.3%	0.59 (0.25-1.25)	0.69 (0.25-1.58)

* Values represent the % in which the alleles appear of the total possible times *(2n)* that they may occur in each group (PH = 102; CP = 148; GAgP = 76); † $\chi^2 = 2.610$, $p > 0.05$; ** Percentage of individuals who carry at least one copy of each polymorphic allele; § $\chi^2 = 1.01$, $p = 0.604$; ⊥ $\chi^2 = 2.12$, $p = 0.345$; *** Not significant (Fisher's exact test).



Graph 1 - Bar chart of the distribution of the TNF-α -308 G/A genotypes in Periodontally Healthy (PH), Chronic Periodontitis (CP), and Generalyzed Aggressive Periodontitis (GAgP) subjects of the study population. No significant differences were observed among groups ($\chi^2 = 2.547$, $p = 0.636$).

were found when only never-smoker and White subjects were included in the analyses (data not shown).

As seen in Graph 1, no significant differences among groups were observed regarding the distributions of the three genotypes AA, AG and GG ($\chi^2 = 2.547$, $p = 0.636$).

Discussion

Periodontal diseases are considered to be complex diseases. Complex diseases are typically polygenic.[12] Disease modifying genes associated with susceptibility and severity of periodontitis have been proposed, but very little is known about which genes may be involved in these diseases.[12] The current study investigated the frequency of the TNF-α -308 G/A alleles and genotypes in Brazilians with different periodontal conditions. Our results showed that the carriage rate of the rare allele A was slightly higher in the PH controls compared to diseased groups, but no significant differences among groups were found. The frequency of allele A varied widely in most of the studies carried out in different populations (range 3% - 32%).[7-9,11,15-17] Despite these differences, our findings are in agreement with the majority of the literature data that failed to show an association of this polymorphism with susceptibility and/or severity of periodontal disease.[7-9,11,15-17]

Regarding the G/G, G/A and A/A genotypes, a similar distribution was observed among the groups as well. Usually, the -308A allele is considered to be the marker of disease susceptibility due to the upregulatory effect of this allele on the cytokine production. However, Folwaczny *et al.*[9] (2004) and Sakellari *et al.*[11] (2006) reported low frequencies for the A/A genotype in periodontitis patients (2% - 4%), whereas this genotype was not detected in periodontally healthy subjects. Other studies did not find the A/A genotype neither in patients nor in controls.[7,17] Our data indicated that the A/A genotype tended to be more prevalent in the PH group (11.6%) in relation to the CP (7.7%) and GAgP (5.6%) groups, although these differences were not significant. Very few studies have reported some correlation between TNF-α polymorphism and periodontitis. Some authors found a significant high frequency of the G/G genotype in severe periodontitis patients,[6,16] whereas Lin *et al.*[5] (2003) reported a significant association between the A allele and moderate-to-advanced periodontitis. Other TNF-α polymorphisms have been

investigated.[15,17] Only Soga *et al.*[17] (2003) showed a significant association between TNF-α -1031, -863 and -857 polymorphisms and severe periodontitis in Japanese individuals.

The inconsistent results observed in the literature could be attributed to several factors related to the definition of disease, population heterogeneity, environmental and demographic confounding risk factors.[12] Although differences in age, ethnicity, gender and smoking history were observed among the subject groups in the current study, the distribution of alleles and genotypes has not changed when the data were independently analyzed in subgroups of patients (data not shown). The sample size in this study was small, thus careful interpretation of the data is necessary. Lack of association between genotypes and clinical *status* may be due to small sample size, particularly for alleles of low prevalence. Nevertheless, we observed a quite elevated carriage rate for the rare allele A compared to other investigations.[7,9,11,17]

Defining a subject population in relation to the type of periodontal disease may also contribute to significant differences among genetic studies. It is likely that overlapping of clinical phenotypes exists between different forms of periodontitis, regardless of the diagnostic criteria used. Even though we used commonly accepted criteria to differentiate clinical groups, comparing our findings with those of other studies is quite difficult due to the different classification systems used.

Finally, the genetic basis for periodontitis may not be related to a single genetic variant, but may be influenced by multiple genes acting synergistically with environmental factors to increase or decrease the likelihood of developing a disease. The TNF cluster region exhibits a high degree of linkage disequilibrium with other polymorphic nearby genes involved in inflammatory responses.[18] For instance, Fassmann *et al.*[8] (2003) suggested that combined genotypes of the TNF-α -308 G/A and LT-α +252 G/A polymorphisms may influence the susceptibility to chronic periodontitis. Thus, the analysis of a single genetic polymorphism might be meaningless for determining a genetic risk factor for periodontal diseases. Instead, combinations of different genotypes for possible gene-gene interactions should be evaluated in these studies.

Conclusion

Based on our results, the TNF-α -308 G/A polymorphism is not associated with increased susceptibility to or severity of periodontitis in this particular Brazilian population.

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