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Investigation of functional gene polymorphisms interleukin-1β, interleukin-6, interleukin-10 and tumor necrosis factor in individuals with oral lichen planus

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Oral lichen planus (OLP) is a chronic inflammatory oral mucosal disease. There are some studies in the literature demonstrating association between cytokines genes polymorphisms and susceptibility to develop some immune-mediate conditions. The purpose of this study was to investigate cytokine gene polymorphisms in a sample of Brazilian patients with OLP. Fifty-three patients with OLP (mean age = 43.1 years; range 20–68 years) and 53 healthy volunteers (mean age = 42.9 years; range 21–67) were genotyped for *IL-1β* +3954 (C/T), *IL-6* −174 (G/C), *IL-10* −1082 (G/A) and *TNFA* −308 (G/A) gene polymorphisms. Statistical analysis was based on the use of logistic regression (*P*-values below 0.05 were considered as significant). *IL-6* and *TNFA* homozygous genotypes were significantly more often detected in OLP patients. These genotypes were associated with an increased risk of OLP development (OR 6.89 and 13.04, respectively). *IL-1β* and *IL-10* gene polymorphisms were not related to OLP development. Our findings clearly demonstrate an association between inheritance of *IL-6* and *TNFA* gene polymorphisms and OLP occurrence, thus giving additional support for genetic basis of this disease.
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Keywords: oral lichen planus; gene polymorphism; genetic; cytokines

Introduction

Lichen planus (LP) is a chronic inflammatory mucocutaneous condition of unknown aetiology. Clinical and immunohistochemical studies (1–3) strongly support a role for immune deregulation in the pathogenesis of oral lichen planus (OLP), specifically involving the cellular arm of the immune system and a complex cytokine network (4, 5). Data from the literature have indicated the importance of NFκβ-dependent cytokines, IL-1β, IL-6 and TNF-α, in the exacerbation and perpetuation of OLP (4, 5). Some authors also believe that IL-10 can inhibit NFκβ activation and that this leads to its anti-inflammatory effects (6–8). Data from the published literature indicate that simultaneous expression of mRNAs for both pro- and anti-inflammatory cytokines occur in OLP and in cultured T-cell lines from the lesion reflecting a complex intralesional environment of counteracting cytokines (9, 10).

Studies *in vitro* and *in vivo* demonstrated that allelic variants of cytokine genes were related to either higher or lower production of cytokines (11, 12) and some authors believe that this genetic variation can even contribute to disease's susceptibility and may influence its course (13, 14). Previous studies regarding OLP (13, 15) and different inflammatory and immune-mediated conditions, such as periodontal disease (16), recurrent aphthous stomatitis (17), graft-versus-host disease (18) and systemic lupus erythematosus (19) showed an association between genetic variations in expression of cytokines and the disease.

Considering that immunological alterations are reported in OLP pathogenesis and IL-1β, IL-6, IL-10 and TNF-α play an important role on immune system regulation (4–8) together with evidence demonstrating that genetic factors are associated with the disease (13, 14, 20), the purpose of the present study was to investigate a possible association between functional polymorphisms related to these cytokines and OLP in a sample of Brazilian patients.

Methods

Subjects and sample collection

Fifty-three subjects affected by OLP and 53 age- and sex-matched control subjects (Table 1) were included in this study. The study protocol was approved by the

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Table 1 Summary of the clinical data of oral lichen planus (OLP) and control patients included in the study

Characteristics	*Values*	
	Control	*OLP*
Age		
Median age years	42	45
Years range	21–67	20–68
Standard deviation	10.6	10.6
Patient gender		
Male, *n* (%)	17 (32.1)	17 (32.1)
Female, *n* (%)	36 (67.9)	36 (67.9)
Clinical OLP		
Reticular *n* (%)	–	44 (83.1)
Erythematous *n* (%)	–	2 (3.7)
Erosive *n* (%)	–	7 (13.2)
Sites committed		
Unique site, *n* (%)	–	16 (30.2)
Multiple sites, *n* (%)	–	37 (69.8)
Dermatological manifestations		
Present *n* (%)	–	6 (11.3)
Absent *n* (%)	–	47 (88.7)
Symptoms		
Present *n* (%)	–	7 (13.2)
Absent *n* (%)	–	46 (86.8)

Institutional Ethics Committee, and informed consent was obtained from the patients.

The patients and controls were recruited from the Oral Diagnosis Clinic, at the Universidade Federal de Minas Gerais. Both the experimental and control groups were of the same geographical area and had identical socio-economic status. Ethnicity was not established, respecting the hazards of judging Brazilians by colour, race and geographical origin (21).

The diagnosis of OLP was confirmed histologically. No patient was suspected to have drug or restoration related lichenoid reaction, and no patient showed histological signs of dysplasia. All patients were asked to inform about OLPs clinical symptoms, and clinically evaluated for dermatological involvement. Exclusion criteria for both groups included the presence of any other significant local or systemic diseases, excluding dental caries. No individual in either group presented chronic periodontitis. Oral mucosa swabs were removed once from the subjects' oral mucosa. The swabs were performed using sterile plastic tips, and placed immediately in Eppendorf microtubes containing 500 µl of Krebs buffer. The pellet was then obtained after 5 min of centrifugation at 13 000 *g* and stored at −20°C until processing.

DNA isolation

DNA extraction was carried out as aforementioned (17). Initially, 450 µl of lyses buffer [6.0 M GuSCN, 65 mM Tris–HCl pH 6.4, 25 mM ethylenediaminetetraacetic acid (EDTA), 1.5% TritonX-100] and 20 µl silica (SiO_2; Sigma S-5631: USB, Cleveland, OH, USA) were added to the microcentrifuge tube containing the oral mucosa swab pellet. The tube was mixed and incubated for 10 min at 56°C, centrifuged at 3000 *g* for 1 min and the supernatant discharged. The pellet with the DNA adsorbed in the silica was washed twice with a 450 µl washing buffer (6.0 M GuSCN, 65 mM Tris–HCl), twice with 70% ethanol, once with a 450 µl acetone, and then dried at 56º C for 10 min. Finally, 100 µl of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) was added and incubated at 56°C for 10 min to elute the DNA. After incubation the solution was vortexed and centrifuged at 5000 *g* for 2 min and approximately 90 µl of supernatant containing DNA was transferred to a new tube. The DNA samples were stored at −20°C until genotyping.

Genotyping

The polymorphisms were assessed by polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers are shown in Table 2. The PCR was carried out in a total volume of 50 µl, containing approximately 400 ng of DNA, primers (20 pmol/reaction), and 25 µl of Pre-mix buffer (Phoneutria Biotecnologia, Belo Horizonte, Brazil). According to the manufacturer, the Pre-mix buffer contained 50 mM KCl, 10 mM Tris–HCl pH 8.4, 0.1% Triton X-100, 1.5 mM $MgCl_2$, deoxynucleoside triphosphates and 1.25 units of *Taq* DNA polymerase. The conditions for amplification consisted of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 54°C for 35 s and 72°C for 30 s.

Table 2 Primer sequence, reference and restriction enzymes used for each polymorphism

Genes	*Primers (references)*	*Restriction enzyme (condition)*	*Genotypes*
IL-1β +3954 (C/T)	5′-CTCAGGTGTCCTCGAAGAAATCAAA-3′ 5′-GCTTTTTTGCTGTGAGTCCCG-3′ (42)	*Taq*I[a] (65°C/4 h)	TT −182 + 12 bp CT −182 + 97 + 85 + 12 bp CC −97 + 85 + 12 bp
IL-6 −174 (G/C)	5′-CAGAAGAACTCAGATGACCTG-3′ 5′-GTGGGGCTGATTGGAAACC-3′ (44)	hsp92II[a] (37°C/8 h)	CC −229 + 122 + 51 + 29 bp GC −229 + 173 + 122 + 51 + 29 bp GG −229 + 173 + 29 bp
IL-10 −1082 (G/A)	5′-CCAAGACAACACTACTAAGGCTCCTTT-3′ 5′-GCTTCTTATATGCTAGTCAGGTA-3′ (45)	XagI[b] (37°C/4 h)	AA −280 + 97 bp GA −280 + 253 + 97 + 27 bp GG −253 + 97 + 27 bp
TNFA −308 (G/A)	5′-AGGCAATAGGTTTTGAGGGCCAT-3′ 5′-TCCTCCCTGCTCCGATTCCG-3′ (37)	NcoI[a] (37°C/12 h)	AA −107 bp GA −107 + 87 + 20 bp GG −87 + 20 bp

[a]Promega, Madison, WI, USA; [b]MBI fermentas

The run was terminated by final elongation at 72°C for 5 min. In all steps the lid temperature was 103°C. The products were digested with restriction enzyme according to the manufacturer's protocols (see Table 2). The visualization of the product was performed in a 6.5% polyacrylamide gel electrophoresis staining with ethidium bromide (0.5 μg/ml).

Statistical analysis

As suggested in the literature for case-control studies (22), we performed initially a chi-square test for trend. The significance of differences in the allele frequencies between the two groups was assessed by use of an approximate binomial proportions test as described before (23, 24).To evaluate the risk of OLP according to single nucleotide polymorphisms, logistic regression models were fitted. The associations were expressed by odd ratios (ORs) and adjusted for age and gender, with the corresponding 95% CIs. The genotypes considered as referents were those associated with less cytokine production in accordance with the literature. The *P*-values < 0.05 were considered significant for both tests. The logistic regression analysis was assessed, using SPSS (SPSS Inc., Chicago, IL, USA), version 14.0, and the univariate analysis were performed using BIOSTAT 3.0 software (Optical Digital Optical Technology, Belém, Brazil).

Results

The distribution of allele frequencies and genotypes of all polymorphisms in patients with OLP and control are shown in Tables 3 and 4. The alleles associated with a higher producer genotype from *IL-6* and *TNFA* polymorphisms were significantly more often detected in OLP subjects (Table 3). A significant increase in the *IL-6* and *TNFA* homozygous genotypes were observed in the group with OLP in the univariate analysis (Table 3). In the multivariate model, adjusted for age and gender, the *IL-6* GG genotype (high producer genotype) represented a sixfold increased risk (OR 6.89) when compared to CC + GC genotype (Table 4). The *TNFA* 'AA' genotype (high producer genotype) was also associated with an increased risk of OLP development (OR 13.04) (Table 4). When we analysed the *TNFA* genotype combinations, we could also observe a statistically significant increased risk for OLP (Table 4). No association between number of lesions, dermatological manifestation or severity of OLP and polymorphisms was observed (data not shown).

Table 3 Distribution of the genotypes and allele frequency in patients with oral lichen planus (OLP) and control subjects

Polymorphism genotype	*OLP (n = 53)* *n (%)*	*Control (n = 53)* *n (%)*	*P-value*
IL-1β +3954(C/T)			
CC CC	31 (58.5)	31 (58.5)	
CT	20 (37.7)	20 (37.7)	
TT	02 (03.8)	02 (03.8)	1.0000[a]
Allele frequency			
C	82 (77.4)	82 (77.4)	
T	24 (22.6)	24 (22.6)	1.0000[b]
IL-6 −174 (G/C)			
CC	01 (01.9)	02 (03.8)	
GC	08 (15.1)	29 (54.7)	
GG	44 (83.0)	22 (41.5)	**< 0.0001**[a]
Allele frequency			
C	10 (09.4)	33 (31.1)	
G	96 (90.6)	73 (68.9)	**< 0.0001**[b]
IL-10 −1082 (G/A)			
AA	20 (37.7)	27 (51.0)	
AG	26 (49.1)	15 (28.3)	
GG	07 (13.2)	11 (20.7)	0.6913[a]
Allele frequency			
A	66 (62.3)	69 (65.1)	
G	40 (37.7)	37 (34.9)	0.6683[b]
TNFA −308 (G/A)			
GG	22 (41.5)	37 (01.9)	
AG	22 (41.5)	15 (28.3)	
AA	09 (17.0)	01 (69.8)	**0.0007**[a]
Allele frequency			
A	40 (37.7)	17 (16.0)	
G	66 (62.3)	89 (84.0)	**0.0004**[b]

A significance level of $P \leq 0.05$ was used
[a]Analysed by chi-square test for trend; [b]analysed by binomial proportions test.

Discussion

The *IL-6* gene is mapped to chromosome 7p21–24 and has an upstream promoter containing 303 bp (25). In 1998, a functional polymorphism in the promoter region of the *IL-6* gene at position −174 (−174 G > C) was identified (26). Many studies have attempted to connect *IL-6* −174 G/C genotype to serum levels. The G > C substitution was found to decrease protein expression by reducing promoter activity (26, 27). An overproduction of IL-6 is involved in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis, Castleman's disease, juvenile idiopathic arthritis and Crohn's disease (28).

Our results showed that OLP patients had a significantly higher frequency of IL-6 −174 polymorphism (G/G) (high producer genotype). This finding does not confirm published data from Carrozzo et al. (13). We might speculate that this disparity might be related to the population heterogeneity. As increased levels of IL-6 are found in the serum and saliva of OLP patients (29, 30), our data support the view that IL-6 may be relevant to OLP pathogenesis. It is well-known that IL-6 is a pro-inflammatory cytokine strongly generated in OLP lesions that has effects on cellular and humoral immunities (29). Its participation on OLP development may include T-cell growth and differentiation, apoptosis of keratinocytes from the basal layer and epithelial proliferation, resulting in hyperplasia (29).

TNF-α is a central mediator of acute inflammation and anti-tumour immunity (31). The gene-encoding TNF-α (*TNFA*) is located in the major histocompatibility complex class III region of chromosome 6 (32). Previous studies described three polymorphic regions in the human TNF-α promoter at −238 (33), −308 (12) and 488 (34) sites, which leads to the substitution of guanine by adenosine in the uncommon alleles. These alterations

Table 4 Association between different polymorphic genotypes and risk to oral lichen planus (OLP) using logistic regression analysis

Polymorphism	*OLP (n = 53)* *n (%)*	*Control (n = 53)* *n (%)*	*OR*[a]	*95% CI*[a] *Min.*	*Max.*	*P-value*
IL-1β +3954 (C/T)						
CC	31 (58.5)	31 (58.5)	Referent			
CT	20 (37.7)	20 (37.7)	0.94	0.35	2.56	0.909
TT	2 (3.8)	2 (3.8)	1.27	0.13	12.37	0.837
CC + CT against TT	51 (96.2) vs. 2 (3.8)	51 (96.2) vs. 2 (3.8)	1.08	0.12	9.92	0.942
CC against CT + TT	31 (58.5) vs. 22 (41.5)	31 (58.5) vs. 22 (41.5)	1.00	0.45	2.28	0.983
IL-6 −174 (G/C)						
CC	1 (1.9)	2 (3.8)	Referent			
GC	8 (15.1)	29 (54.7)	0.61	0.04	8.51	0.712
GG	44 (83.0)	22 (41.5)	3.99	0.31	50.86	0.286
CC + CG against GG	9 (17) vs. 44 (83)	31 (58.5) vs. 22 (41.5)	6.89	2.67	17.76	**0.000**
CC against GC + GG	1 (1.9) vs. 52 (98.1)	2 (3.8) vs. 51 (96.2)	NA	–	–	–
IL-10 −1082 (G/A)						
AA	20 (37.7)	27 (51)	Referent			
AG	26 (49.1)	15 (28.3)	2.21	0.79	6.20	0.133
GG	7 (13.2)	11 (20.7)	0.79	0.22	2.84	0.717
AA + AG against GG	46 (86.8) vs. 7 (13.2)	42 (79.3) vs. 11 (20.7)	0.56	0.17	1.83	0.335
AA against AG + GG	20 (37.7) vs. 33 (62.3)	27 (51) vs. 26 (49)	1.73	0.77	3.88	0.186
TNFA −308 (G/A)						
GG	22 (41.5)	37 (69.8)	Referent			
GA	22 (41.5)	15 (28.3)	2.51	0.92	6.85	0.073
AA	9 (17)	1 (1.9)	13.04	1.29	131.37	**0.029**
GG + GA against AA	44 (83) vs. 9 (17)	52 (98.1) vs. 1 (1.9)	9.41	1.03	85.85	**0.047**
GG against GA + AA	22 (41.5) vs. 31 (58.5)	37 (69.8) vs. 16 (30.2)	3.27	1.45	7.34	**0.004**

A significance level of $P \leq 0.05$ was used.
CI, confidence interval; NA, not applicable.
[a]Adjusted for gender and age.

that can regulate gene expression have been associated with inflammatory and malignant conditions (35). Data from literature demonstrate that the allele at −308A is associated with high TNF-α production (36, 37).

Our results demonstrated that OLP patients had a significantly higher frequency of *TNFA −308* polymorphism (A/A), which is associated with a higher cytokine production (38, 39). Krasowska et al. (15) did not find any correlation between *TNFA* genotype distribution, allele frequency and LP. On the other hand, our findings are in accordance with Carrozzo et al. (13), who observed that −308A TNF-α allele was more frequent in OLP patients. Significantly, other studies indicated a high level of TNF-α protein in involved oral mucosa (40), in the serum (41) and in the oral fluids of OLP patients (42). Carrozzo et al. (13) also observed that OLP patients with skin involvement presented the −308 *TNFA* G/A genotype with significantly higher percentage (82%) than patients with exclusive oral lesions (30%), suggesting that the simultaneous presence of cutaneous lesions could be caused by higher TNF-α production. The low number of individuals with dermatological manifestations in our study made this association difficult to be demonstrated. Taken together, all these data support the idea that TNF-α plays a key regulatory role in the initiation and progression of OLP. Our results also confirmed that *IL-1β* and *IL-10* gene polymorphisms are not related to OLP development (13).

IL-1β, IL-6, IL-8 and TNF-α are NFκβ dependent cytokines which play an important role on OLP pathogenesis (4, 5). The treatment of OLP with dexamethasone reduces these pro-inflammatory cytokine levels in saliva (43). The effect of this treatment over individuals with distinct cytokines genotypes seems to be an interesting target for further studies.

There is an important limitation to the present study that should be considered. As our investigation was restricted to a genetic analysis, no speculation could be performed about the functional relations between cytokines, such as synergistic effects. In conclusion, our findings clearly demonstrate an association between inheritance of *IL-6* and *TNFA* gene polymorphisms and OLP occurrence, thus giving additional support for genetic basis of this disease.

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