Investigation of functional gene polymorphisms IL-1β, IL-6, IL-10 and TNF-α in individuals with recurrent aphthous stomatitis

André Luiz Sena Guimarães, Jeane de Fátima Correia-Silva, Alessandra Rosa de Sá, Junia Maria Netto Vitória, Marina Gonçalves Diniz, Fernando de Oliveira Costa, Ricardo Santiago Gomez*

Department of Oral Surgery and Pathology, School of Dentistry, Universidade Federal, de Minas Gerais, Belo Horizonte, Brazil

Abstract

Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration in an otherwise healthy individual. Some reports in the literature indicate that RAS may have immunological, psychological, genetic and microbiological bases.

Objective: The purpose of the present study was to investigate, using binary logistic regression analyses, a possible association between the functional IL-1β +3954 (C/T), IL-6 −174 (G/C), IL-10 −1082 (G/A) and TNF-α −308 (G/A) genetic polymorphism and RAS in a sample of Brazilian patients, using a multivariate statistical analysis.

Design: Sixty-four consecutive subjects affected by minor and major forms of RAS and 64 healthy volunteers were genotyped. To investigate the association between the single nucleotide polymorphisms and risk of RAS, binary logistic regression models were fitted. The associations were expressed by odd ratios (ORs) and adjusted for age and gender, with the corresponding 95% CIs. P-values less than 0.05 were considered significant.

Results: A significant increase in the IL-1β and TNF-α heterozygous genotypes were associated with an increased risk of RAS development (OR 2.40 and 3.07, respectively), in the multivariate model.

Conclusion: Our findings demonstrate that polymorphisms of high IL-1β and TNF-α production were associated with an increased risk of RAS development. Our findings also give additional support to a genetic basis for RAS pathogenesis.

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1. Introduction

Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration in an otherwise healthy individual.¹ Clinically, RAS includes three different variants, minor aphthous ulcers, major aphthous ulcers and herpetiform ulcers.²,³ It has been estimated that 20% of the general population will suffer from RAS at some time in their lives.²,⁴ Although the aetiology of RAS is unknown, current evidence indicates the presence of genetic factors in this disease. This has been confirmed by the finding that more than 40% of patients may have a family history of RAS.⁵ The probability of a sibling developing RAS may also be influenced by the parents’ RAS status.⁶ Moreover, there is
a high correlation of RAS in monozygote but not in dizygote twins.7

Psychological factors including anxiety and stress were associated with RAS in a considerable number of studies,5,8 thus supporting the findings of our previous study in which RAS patients showed an increased frequency of serotonin transporter gene polymorphism (5-HTTLPR) associated with anxiety-related traits.9

Some forms of immune dysfunction appear to be related to RAS pathogenesis. An increased local expression of Th1 genes10 and systemic production of cytokines, such as IL-2, TNF-α and IL-6 by peripheral blood mononuclear cells were observed in RAS patients.11 In addition, decreased IL-10 mRNA levels were reported in RAS patients, which suggests a failure of the immune system to suppress inflammatory reaction to oral mucosa.12

A large number of functional genetic polymorphisms in the immunological system have been described in previous literature. Some genotypes of these polymorphisms can increase protein production13,14 while other polymorphisms, such as IL-1β +3954 (C/T), were associated with RAS in a sample of British patients.15 Considering that immunological alterations are reported in RAS pathogenesis, together with evidence demonstrating that genetic factors are associated with the disease, the purpose of the present study was to investigate a possible association between the functional IL-1β +3954 (C/T), IL-6 –174 (G/C), IL-10 –1082 (G/A) and TNF-α –308 (G/A) genetic polymorphisms and RAS in a sample of Brazilian patients.

2. Material and methods

2.1. Subjects and sample collection

Sixty-four consecutive subjects affected by minor and major forms of RAS (Table 1) and 64 age and sex matched control subjects (mean age = 36.9 years; range 8–84 years; standard deviation 16.5 years) were included in this study. The patients were recruited from the Oral Diagnosis Clinic at the Universidade Federal de Minas Gerais. Both the experimental and control groups were of the same geographic area and had identical socio-economic status. Ethnicity was not established, respecting the hazards of judging Brazilians by color, race and geographical origin as demonstrated in the past findings.16

The diagnosis of RAS was based on accepted clinical criteria.17 The control group was comprised of patients with no history of RAS nor systemic diseases. 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. The study protocol was approved by the local Ethics Committee and informed consent was obtained from either the patients or the legal guardian when the patient was less than 18 years of age.

Oral mucosa swabs were removed once from the subjects’ buccal 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.

2.2. DNA isolation

DNA extraction was carried out as aforementioned.18 Initially, 450 µl of lyses buffer (6.0 M GuSCN, 65 mM Tris–HCl pH 6.4, 25 mM EDTA, 1.5% Triton X-100) and 20 µl silica (SiO2, Sigma S-5631) 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 56 °C for 1 min. Finally, 100 µl of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) were 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.

2.3. 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 MgCl2, 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. 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 manufacturer protocols (see Table 2).
visualization of the product was performed in a 6.5% polyacrylamide gel electrophoresis staining with ethidium bromide (0.5 μg/ml).

2.4. Statistical analysis

The univariate analyses were performed using the Fisher exact test or the Chi-square test. To investigate the association between the single nucleotide polymorphisms and risk of RAS, binary logistic regression models were fitted. The associations were expressed by odds ratios (ORs) and adjusted for age (<26 and ≥26 years) and gender, with the corresponding 95% CIs. P-values less than 0.05 were considered significant. The multivariate analyses were assessed using SPSS (SPSS Inc., Chicago), version 14.0, and the univariate analyses were performed using BioStat 3.0 software (Optical Digital Optical Technology, Belém, Brazil).

3. Results

The distribution of genotype and allele frequencies of all polymorphisms in patients with RAS and control are shown in Tables 3 (univariate analyses) and 4 (multivariate analyses). A significant increase in the IL-1β and TNF-α heterozygous genotypes was observed in the group with RAS in the univariate analysis (P=0.03 and 0.04). In the multivariate model, adjusted for age and gender, the same genotypes of IL-1β and TNF-α shown were associated with an increased risk of RAS development (OR 2.40 and 3.07, respectively).

4. Discussion

RAS is a very common oral disease of unknown etiology. Many local and systemic factors have been associated with the condition. Some reports in the literature indicate that RAS may have immunological, psychological, genetic and microbiological bases.1,3,5,17 Although studies have tried to identify the role of the immune system in RAS, the immunopathogenesis remains to be established.19,20 Evidence suggests that the ulceration is the result of an abnormal cytokine cascade in the oral mucosa that leads to enhanced cell-mediated immune response directed toward focal areas of the oral mucosa.10,12 Recent scanning with cDNA microarray analyses in RAS showed a more intense activity of the Th1 gene cluster relative to the Th2 gene cluster.19 Polymorphisms associated with cytokines have been used to investigate the pathogenesis of various diseases. In the current study, the univariate analysis showed that the polymorphism CT at IL-1β+3954 was associated with RAS. In the multivariate analysis, we observed that this genotype was associated with an increased risk of RAS development (OR 2.40). Previous literature has proven that this genotype has been associated with two-fold IL-1β production.13 One such study, using a univariate analysis, also showed an increased frequency of IL-1β+3954 and -511 polymorphisms

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers (references)</th>
<th>Restriction enzyme (condition)</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β +3954 (C/T)</td>
<td>5’-CTCAGGTGTCCTCGAAGAAATCAA-3’ 5’-GCTTTTTTGTGTGAGTCCCG-3’ (Pociot et al.13)</td>
<td>Taq1a (65/4 h)</td>
<td>TT–182 + 12 bp CT–182 + 97 + 85 + 12 bp CC–97 + 85 + 12 bp</td>
</tr>
<tr>
<td>IL-6 –174 (G/C)</td>
<td>5’-CGAGAAGACTCAGATGACCTG-3’ 5’-GGGGGTCGGTGGAAACC-3’ (Klein et al.25)</td>
<td>hsp92IIa (37/4 h)</td>
<td>CC–229 + 122 + 51 + 29 bp CG–229 + 173 + 29;bp GG–229 + 122 + 51 + 29 bp</td>
</tr>
<tr>
<td>IL-10 –1082 (G/A)</td>
<td>5’-CCAAGACACACTAAGGCCTCCTT-3’ 5’-GGCTCTATATGCTAGTGCAGTA-3’ (Koch et al.26)</td>
<td>Xagb (37/8 h)</td>
<td>AA–280 + 97 bp GA–280 + 253 + 97 + 27 bp GG–253 + 97 + 27 bp</td>
</tr>
<tr>
<td>TNF-α –308 (G/A)</td>
<td>5’-GAACCAATAGGGTTGAGGCCC-3’ 5’-TCCTCCCTGCCTCGGATTCC-3’ (Wilson et al.14)</td>
<td>Ncfoa (37/12 h)</td>
<td>AA–107 bp GA–107 + 87 + 20 bp GG–87 + 20 bp</td>
</tr>
</tbody>
</table>

a Promega, Madison, USA.
b MBI Fermentas.

Table 3 – Distribution of the genotypes in patients with recurrent aphthous stomatitis (RAS) and control subjects using univariate analyses

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>RAS (N = 64)</th>
<th>Control (N = 64)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β +3954 (C/T) (N, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>28 (56.2)</td>
<td>41 (64)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>36 (43.8)</td>
<td>23 (36)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.03</td>
</tr>
<tr>
<td>IL-6 –174 (G/C) (N, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1 (1.6)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>25 (39)</td>
<td>24 (37.5)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>38 (59.4)</td>
<td>40 (62.5)</td>
<td>0.58</td>
</tr>
<tr>
<td>IL-10 –1082 (G/A) (N, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>31 (48.4)</td>
<td>31 (48.4)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>26 (40.6)</td>
<td>23 (36)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>7 (11)</td>
<td>10 (15.6)</td>
<td>0.70</td>
</tr>
<tr>
<td>TNF-α –308 (G/A) (N, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>38 (59.4)</td>
<td>47 (73.4)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>22 (34.4)</td>
<td>10 (15.6)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>4 (6.2)</td>
<td>7 (11)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

P-values from Chi-squared test. A significance level of P ≤ 0.05 was used.
associated with a high cytokine producer genotype on RAS subjects.\textsuperscript{15}

The relation between local damage in RAS and IL-1\textsuperscript{b} can be explained by the action of this cytokine as a primary activator of early chemotactic cytokines, as well as of the expression of endothelial cell adhesion molecules which facilitate the migration of leucocytes into tissues.\textsuperscript{21,22} Increased levels of vascular adhesion molecule-1 (VCAM-1) can be observed in the sera of RAS patients.\textsuperscript{23} Moreover, a recent study demonstrated a higher IL-1\textsuperscript{b} transcription in RAS lesions as compared to normal mucosa.\textsuperscript{10} Therefore, we may speculate that the expression of the adhesion molecules may be induced by increased levels of IL-1\textsuperscript{b} present in the individuals with the high producer genotype. Although RAS patients showed a genotype associated with high IL-1\textsuperscript{b} production, other authors have shown that IL-1\textsuperscript{b} production by peripheral blood mononuclear cells was not increased in RAS.\textsuperscript{11}

Another mechanism to explain IL-1\textsuperscript{b} polymorphism and RAS development may also be considered. It is well-known that psychological factors are implied in the pathogenesis of RAS.\textsuperscript{8} A previous study conducted by our group showed that RAS patients showed an increased frequency of serotonin transporter gene polymorphism (5-HTTLPR) associated with anxiety-related traits.\textsuperscript{9} As IL-1\textsuperscript{b} levels have proven to be higher in the blood or cerebrospinal fluid of depressed patients,\textsuperscript{24,25} the high producer IL-1\textsuperscript{b} genotype may be more susceptible to depression and RAS. It is interesting to note that IL-1\textsuperscript{b} polymorphism has been related to psychosis susceptibility and early development of Alzheimer’s disease.\textsuperscript{26}

In the current study, we observed that TNF-\textalpha intermediary producer genotype was associated with an increased risk of RAS (OR = 3.07). The low number of individuals in case and control groups with a high TNF-\textalpha producer genotype may explain why this genotype was not associated with RAS. No previous studies demonstrate the association of TNF-\textalpha – 308 (G/A) polymorphism and RAS.\textsuperscript{27} TNF-\textalpha does indeed present some important immune regulatory activities and studies have suggested its relation to RAS. Likewise, elevated levels of TNF-\textalpha have been reported in the lesional mucosa and in the peripheral blood of RAS patients.\textsuperscript{11,12,19,28} Enhanced cytotoxic destruction of epithelial cells by TNF-\textalpha produced by peripheral blood mononuclear cells\textsuperscript{29} and leucocytes\textsuperscript{28} in RAS subjects was demonstrated by in vitro studies. Moreover, RAS can be prevented by endogenous TNF-\textalpha synthesis inhibitors, such as thalidomide\textsuperscript{30} and pentoxifylline.\textsuperscript{19}

Although IL-6 and IL-10 were implied in RAS pathogenesis,\textsuperscript{11,12,31} our data shows that polymorphisms on these genes were not associated with RAS. This is in contradiction to a previous study that demonstrated an association between IL-6 polymorphism and RAS.\textsuperscript{27} This disparity is probably related to population heterogeneity. In conclusion, our findings demonstrate that polymorphisms of high IL-1\textsuperscript{b} and TNF-\textalpha production were associated with an increased risk of RAS development. Our findings also give additional support to a genetic basis for RAS pathogenesis.

**Acknowledgements**

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**References**