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Serotonin transporter gene polymorphism (5-HTTLPR) in patients with recurrent aphthous stomatitis

Junia Maria Netto Victoria[1], Jeane de Fátima Correia-Silva[1], Flávio Juliano Pimenta[1], Evanguedes Kalapothakis[2], Ricardo Santiago Gomez[1]

[1]*Department and Oral Surgery and Pathology, School of Dentistry and* [2]*Department of Pharmacology, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil*

BACKGROUND: Considerable evidence indicates that serotonergic mechanisms, particularly the serotonin transporter (5-HTT) may be involved in psychological alterations. Recent findings have demonstrated that depression and stress are influenced by polymorphism of the promoter region of 5-HTT (5-HTTLPR) and that the short allele (*S*) is associated with reduced transcriptional efficiency resulting in reduced serotonin expression and uptake. As psychological and genetic factors have been implicated in the pathogenesis of recurrent aphthous stomatitis (RAS), the purpose of the present study was to investigate 5-HTTLPR polymorphism in patients with RAS compared with control subjects.

METHODS: Sixty-nine consecutive subjects affected by minor and major forms of RAS and 70 healthy volunteers were genotyped at 5-HTTLPR. The chi-square test was used for statistical analysis.

RESULTS: A significant increase in the genotype of SS ($P = 0.05$) and of the allele *S* ($P = 0.04$) in the group of RAS were observed.

CONCLUSION: Our findings demonstrate that RAS patients have a tendency to show polymorphism associated with anxiety-related traits.

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Introduction

Recurrent aphthous stomatitis (RAS) is the most common oral mucosal disease and has been the subject of many clinical and research studies. It is characterized by periodic painful, single or multiple ulcers that heal spontaneously. On the basis of size, number of ulcers and features of the healing process, RAS lesions have been classified into three groups, namely minor, major and herpetiform ulcerations (1). The precise aetiology and pathogenesis of RAS remains unclear and several factors as nutrition, drugs, food hypersensitivity, hormones, infection, trauma, tobacco and psychological stress are generally considered important to the development of RAS (2, 3). There are a considerable number of studies suggesting an association between RAS and psychological factors including anxiety and stress (3).

Some studies have suggested that sequence variability in the genes of the serotonergic neurotransmission system may be responsible for a portion of the expression of externalizing behaviours mediated by this system. In particular, a large number of studies have focused on the serotonin transporter (5-HTT), which is the target site for the selective serotonin reuptake inhibitors such as fluoxetine and paroxetine (4). The gene for 5-HTT (*SLC6A4*) is located on chromosome 17q12 and consists of a promoter and 14 exons spanning 31 kb (5, 6). Two polymorphic regions of *SLC6A4* have been identified including a 44 bp insertion–deletion in the promoter region (5-HTTLPR) and a 17 bp variable number of tandem repeats (VNTR) in the second intron of the gene (6, 7). Two alleles, termed long (*L* with 16 repeats) and short (*S* with 14 repeats) are found in the first polymorphism (5-HTTLPR) (6). Recently, it was demonstrated that *L* and *S* variants of the promoter polymorphism differentially modulate transcription of *SLC6A4*, with the *S* variant being less efficient (8). Some studies have suggested a possible involvement of the 5-HTTLPR genotype with suicidal behaviour (9–11), depression (12), anxiety (13) and alcoholism (14).

As psychobiological factors have been implicated in the pathogenesis of RAS, the purpose of the present study was to investigate the 5-HTTLPR polymorphism of the 5-HTT gene in patients with RAS compared with control subjects.

Correspondence: Prof. Ricardo Santiago Gomez, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, Belo Horizonte-MG, Brazil CEP 31270-901. Tel.: +55-31-3499-2477. Fax: +55-31-3499-2472. E-mail: rsgomez@ufmg.br
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Material and methods

Subjects and sample collection

Sixty-nine consecutive subjects (age 7–69) affected by minor and major forms of RAS and 70 healthy volunteers were included in the study and were recruited from the Oral Diagnosis Clinic at the Universidade Federal de Minas Gerais. Both the experimental and control groups lived in same geographic area, had the same socio-economic status and were age- and gender-matched. Ethnicity was not established as the hazards of judging Brazilians by colour, race and geographical origin have been recently demonstrated (15).

The diagnosis of RAS was based on accepted clinical criteria (16). The control group was composed of patients without any history of RAS. Exclusion criteria for both groups were the presence, apart from dental caries or chronic adult periodontal disease, of any other significant local or systemic diseases. The study protocol was approved by local Ethical Committee and informed consent was obtained from all patients or from the parents when < 18 years.

Oral mucosa swabs were taken once from the subjects on the buccal mucosa. The swabs were performed with sterile plastic tips, placed immediately in Eppendorf microtubes containing 500 µl of Krebs buffer (NaCl 20%, KCl 2%, $CaCl_2 2H_2O$ 2%, $MgSO_4$, KH_2PO_4, $C_6H_{12}O_6$), and the pellet obtained after 10 min of centrifugation at 17 900 *g* was stored at −20°C until processing.

DNA isolation

The DNA extraction was carried out as described by Boom et al. (17) and modified as below. We added 450 µl of lyses buffer [6.0 M GuSCN, 65 mM Tris-HCl pH 6.4, 25 mM ethylenediaminetetraacetic acid (EDTA), 1.5% Triton X-100] and 20 µl silica (SiO_2; Sigma S-5631: USB, Cleveland, OH, USA) to the microcentrifuge tube containing the oral mucosa swab pellet. The tube was vortexed and incubated for 10 min at 56°C, centrifuged at 3000 *g* for 1 min and the supernatant discharged. The pellet obtained (DNA adsorbed to the silica) was washed twice with 450 µl washing buffer (6.0 M GuSCN, 65 mM Tris-HCl), twice with 70% ethanol, once with 450 µl acetone and dried at 56°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 homogenized and centrifuged at 5000 *g* for 2 min and the supernatant containing DNA transferred to a new tube.

Genotyping

The insertion/deletion in the *5-HTT* gene-linked polymorphic region (5-HTTLPR) was amplified with primers 5′-CCGCTCTGAATGCCAGCACCTAAC-3′ and 5′-AGAGGGACTGAGCTGGACAACCAC-3′ (6). After DNA extraction 2 µl of DNA solution were used for polymerase chain reaction (PCR). Samples were subjected to 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 68°C and 45 s at 72°C. The run was terminated by a 5 min elongation step at 72°C. PCR was carried out in a 50 µl mixture containing *Taq* DNA polymerase (1 unit/reaction), PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.4, 1.5 mM $MgCl_2$), deoxynucleoside triphosphates (0.1 mM/reaction of each dNTP) and primers (20 pmol/reaction). All samples were amplified using a DNA thermal cycler (Programmable Thermal Controller, PTC). Allele sizes were determined by comparison of bands with size standards after electrophoresis in a 6.5% native polyacrylamide gel and silver staining. Amplification of the 5-HTTLPR gave two alleles differing by 44 bp (*L* and *S*; Fig. 1).

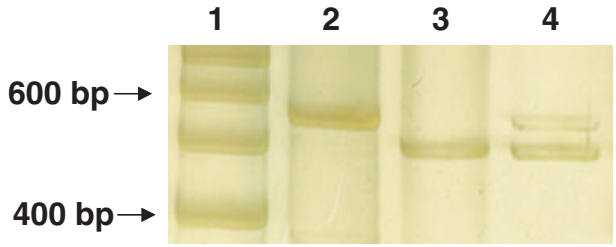

Figure 1 Electrophoresis in a 6.5% polyacrylamide gel. Lane 1, ladder 100 bp; lane 2, genotype LL; lane 3, genotype SS; lane 4: genotype LS.

The *L* and *S* alleles were also verified in some cases by DNA sequencing. DNA sequencing reactions were performed on both strands using the chain termination method (18). PCR products were purified using GFX PCR and Gel Band Purification Kit (Amersham Bioscience, Amersham, UK), and sequenced using BigDye Terminator reaction chemistry kit on an ABI PRISM 310 Genetic Analyzer (ABI Applied Biosystem, Foster City, CA, USA). The sequences were compared with sequences in the GenBanK databases (19) (Fig. 2).

Statistical analysis

Statistical significance of differences between case and control group distributions for alleles and genotypes were determined using chi-squared test. A significance level of $P \leq 0.05$ was used. The observed genotype frequencies were compared with those calculated from Hardy–Weinberg equilibrium theory. All the statistical analyses were performed using BIOSTAT 3.0 software (Optical Digital Optical Technology, Belém, Brazil).

Results

The distribution of genotype and allele frequencies of 5-HTTLPR polymorphism in patients with RAS and control are shown in Table 1. A significant increase in the genotype of SS ($P = 0.05$) and of the allele *S* ($P = 0.04$) in the group of RAS were observed. The observed distribution of 5-HTTLPR genotypes in the patient group (LL:LS:SS, 17:26:26) was statistically different from those (13:34:22) expected from the Hardy–Weinberg equilibrium equation ($P = 0.05$).

Discussion

The RAS is a very common oral disease of unknown aetiology. Many local and systemic factors have been

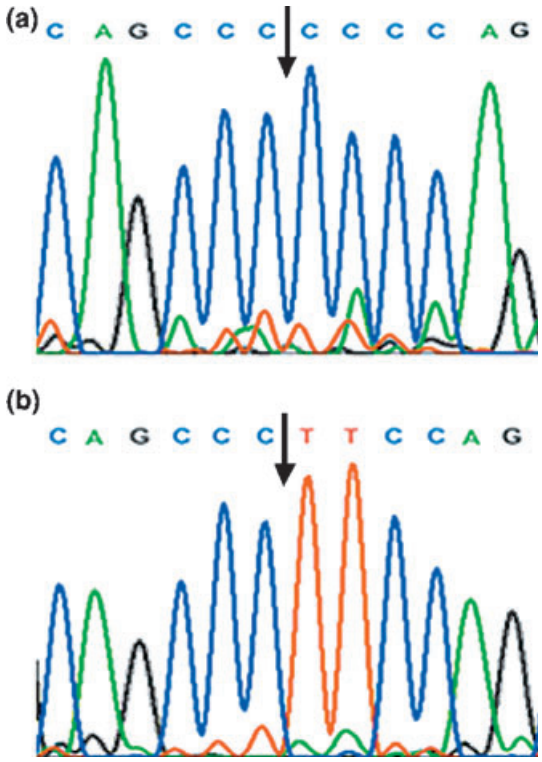

Figure 2 DNA sequencing of genotypes LL and SS in recurrent aphthous stomatitis (RAS) patients samples. (a) Representative sequencing of genotype LL. (b) Representative sequencing of genotype SS. ↓ represents the site that occur a 44 bp deletion on allelic *S* in the promoter region of gene for *5-HTT* (*SLC6A4*).

Table 1 Distribution of genotype and allele frequencies (%) of 5-HTTLPR in patients with recurrent aphthous stomatitis (RAS) and control subjects

	RAS (n = 69)	*Control (n = 70)*	*P-value*
Genotypes, *n* (%)			
LL	17 (24.6)	26 (37.2)	
LS	26 (37.7)	30 (42.8)	0.05*
SS	26 (37.7)	14 (20.0)	
Alleles (%)			
L	43.0	58.0	
S	57.0	42.0	0.04**

*χ^2 value = 5.763, **χ^2 value = 4.500.

associated with condition. Some reports in the literature indicate that RAS may have an immunological, psychiatric, genetic and microbiological bases (1–3, 20–24). Studies have been carried out trying to identify the stress, anxiety and depression levels in patients with RAS by means of diverse questionnaires, such as the Hamilton Anxiety Depression (HAD) Scale, Hamilton Anxiety Scale and others (23). Despite contradictory results, psychological factors have been considered relevant to the pathogenesis of RAS (23, 25, 26). To date no study has attempted to investigate a genetic factor associated with psychological behaviour in a set of RAS patients.

The *5-HTT* regulates the magnitude and duration of serotonergic neurotransmission and polymorphism in this gene was found to be associated with anxiety-related traits (8, 27). The 5-HTTLPR have been extensively examined and *in vitro* studies have demonstrated that the *S* allele has been associated with reduced transcriptional efficiency resulting in reduced serotonin expression and uptake (8).

The results demonstrated an increased frequency of the genotype SS and of the allele *S* on RAS patients compared with control subjects ($P = 0.04$). Furthermore, in contrast to control, the genotypes in the patient group were not distributed according to the Hardy–Weinberg equilibrium. These findings are in accordance to clinical studies that suggest association between psychological disorders and RAS and may also help to explain the familial basis of RAS. As almost of patients included had the minor form of RAS, no attempt was made to compare the polymorphism between different clinical types of the disease. Despite the small sample size, the distribution of the normal genotypes in the present study was similar to the distribution in a larger study of 505 subjects (8). In conclusion, our findings demonstrate that RAS patients have a tendency to show polymorphism associated with anxiety-related traits. The 5-HTTLPR polymorphism may be an interesting candidate for future studies on RAS pathogenesis.

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