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Investigation of *CD40* gene *rs4810485* and *rs1883832* mutations in patients with recurrent aphthous stomatitis

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ABSTRACT

Objectives: Recurrent aphthous stomatitis (RAS) is a common painful disorder affecting oral health, mucosa and overall quality of life. The etiopathogenesis of RAS remains unclear. RAS shows a large genetic diversity among the patients. In present study, we investigated whether *CD40* gene *rs4810485* and *rs1883832* are associated with RAS and its clinical findings in Turkish patients.
Materials and methods: Genomic DNA obtained from 387 individuals (160 patients with RAS and 227 healthy controls) were used in the study. *CD40* gene *rs4810485* and *rs1883832* mutations were determined by using polymerase chain reaction with the specific primers.
Results: There was no statistically significant difference between the groups with respect to genotype and allele distribution ($p > 0.05$, OR 0.94, 95% CI 0.70–1.28, OR 1.01 95% CI 0.75–1.37, respectively). Additionally, there was no statistically significant difference in the combined genotype analysis of *CD40* gene *rs4810485* and *rs1883832* mutations ($p > 0.05$).
Conclusions: According to our results, we found that *CD40* gene mutations are not associated with RAS. We are convinced that *CD40* gene mutations do not predispose to develop RAS in Turkish population. To our knowledge, this is the first study regarding *CD40* gene *rs4810485* and *rs1883832* mutations investigated in RAS patients.

1. Introduction

Recurrent aphthous stomatitis (RAS) is a common painful disorder affecting oral health, mucosa and overall quality of life. Hitherto, this disease has been reported in approximately 20% of the world population (Albanidou-Farmaki et al., 2008 ;Bazrafshani, Hajeer, Ollier, & Thornhill, 2002; Freysdottir, Lau, & Fortune, 1999). RAS has been described under three different clinical conditions as minor RAS, major RAS and herpetiform lesions with multiple minor aphthous ulcers seen in the majority of RAS patients (Bazrafshani et al., 2002; Scully & Porter, 2008; Slebioda, Szponar, & Kowalska, 2013). The etiopathogenesis of RAS remains unclear. Many local and systemic factors could be involved in the etiopathogenesis of RAS ulcerations such as viral and bacterial infections (e.g. oral streptococci, helicobacter pylori), food hypersensitivity, psychological stress, genetic factors (e.g. human leukocyte antigens (HLA) and distinct racial and ethnic origins), and systemic diseases (e.g. Behcet's syndrome and acquired immune deficiency syndrome (AIDS) (Lewkowicz et al., 2003)). Additionally, the familial accumulation was observed in up to 40% of patients with RAS (Guimarães et al., 2007).

RAS belongs to autoinflammatory disease group, arising from abnormal immune interaction on the oral mucosal cells. The impaired activation of the immune system components such as tumor necrosis factors leads to development of RAS (Antoniades, Bakogiannis, Tousoulis, Antonopoulos, & Stefanadis, 2009; Lewkowicz et al., 2003). It was reported that the possible mechanism for RAS development involves tissue-specific autoimmunity. Also, there is a theory suggesting that polymorphonuclear leukocytes play a role in the pathogenesis of RAS (Lewkowicz et al., 2003). Hence, T cell-mediated immune processes are responsible for a significant part of the immune-mediated pathogenesis in RAS. The development of the RAS is due to not only local reactions, but also

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systemic conditions. It was reported that the number of CD4+ T cells and CD8+ T cells showed a decrease and increase in RAS patients, respectively (Freysdottir et al., 1999). It was also stated that tumor necrosis factor alpha (TNF-α) is effective in the T cell-mediated immune response. TNF-α is a major pro-inflammatory cytokine that has a chemotactic effect on systemic immune reaction components such as the neutrophils and the major histocompatibility complexes (MHC) (Scully & Porter, 2008). TNF family, along with inflammatory cytokine, is responsible for development of new aphthous ulcers in patients with RAS (Antoniades et al., 2009).

CD40 is a member of the TNF superfamily, and the gene encoding it is located on the chromosome 20q12-q13.2. *CD40* expression is induced by pro-inflammatory stimuli such as interleukin (IL) 1, 3, 4, TNF-α and interferon-γ (IFN). It also has an impact on humoral and cellular immune response. *CD40* expression is activated through *CD40*/*CD40L* ligation and the receptor is internalized into the cell (Antoniades et al., 2009). *CD40* expression affects basal and parabasal oral keratinocyte activities, but it is absent in oral epithelial dysplasia and T cell mediated conditions are downregulated by keratinocyte proliferation (Villarroel, Speight, & Barrett, 2006). *CD40*/*CD40* ligand interaction leads to autoimmune disease by creating a domino effect on the immune system component. Therefore, we think that the role of *CD40* should be investigated with regard to development of oral ulcerations in patients with RAS. While CD154/*CD40L* binds to *CD40*, it initiates the release of cytokines and expression of cell adhesion molecules, and different inflammatory processes are triggered (García-Bermúdez et al., 2012). To our knowledge, *CD40* gene *rs4810485* and *rs1883832* regions mutations in RAS have not been studied previously. The purpose of the present study was to investigate a possible association between the functional *CD40* gene *rs4810485* and *rs1883832* regions mutations with RAS susceptibility and disease progression in Turkish RAS patients.

2. Materials and methods

2.1. Subjects

This study is a case-control study and included 160 patients with RAS (mean age 35.69 ± 10.735 SD years) and 227 healthy controls (mean age 37.18 ± 11.910 SD years). All patients and control subjects were studied in Gaziosmanpasa University, Faculty of Dentistry, Department of Maxillofacial Surgery and Department of Prosthodontics clinics in Tokat, Turkey. They were enrolled into this study according to the clinical criteria (Ship, Chavez, Doerr, Henson, & Sarmadi, 2000). The exclusion criteria were established according to Behcet's disease, Celiac disease and other gastrointestinal symptoms or diseases. Informed consent was obtained in accordance with the study protocol, approved by the ethics committee of Medical Faculty (15-KAEK-080). All patients signed a written consent form after being informed about the details of the study. A complete clinical evaluation was done for all patients. The controls were selected by excluding the diagnosis of RAS. All the individuals in the control group were healthy. Data collection sheet included information such as gender, age, age at disease onset, family history of RAS, presence of papulopustules, systemic involvement, erythema nodosum, genital ulcers and pathergy positivity. Characteristics of the patients with RAS were summarized in Table 1.

2.2. Genotype determination

Genomic DNA was extracted from ethylendiamine-tetraacetate (EDTA)-treated whole venous blood samples using a commercial DNA isolation kit (Sigma-Aldrich, Taufkirchen, Germany) and stored at −20 °C. The *CD40* gene *rs4810485* mutation was analyzed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) methods. The PCR protocol was consisted of an initial melting step of 2 min at 94° C; followed by 35 cycles of 1 min at 94° C, 1 min at 60° C, and 1 min at 72° C; and a final elongation step of 7 min at 72° C. PCR primers (*F:5′*-TATTTTTGTAGTTCCTCATTCTG-*3′* and R:5′-GCCCCCCTTTACCTCTTTC-3′) were used to amplify a portion of the *CD40* gene from 100 ng of genomic DNA, 1 × PCR buffer, 200 μm dNTP, 0.1 μm each primers, and 1 U of Taq DNA polymerase (Invitrogen, GM008-1000-5). After amplification, the 10 μl PCR product was digested with *MspI* (Fermentas, FD0544) at 37° C 5 min. (digested products 184 bp and 104 bp).

The *CD40* gene *rs1883832* mutation was analyzed by PCR based RFLP methods. The PCR protocol was consisted of an initial melting step of 2 min at 94° C; followed by 35 cycles of 1 min at 94° C, 1 min at 60° C, and 1 min at 72° C; and a final elongation step of 7 min at 72° C. PCR primers (F:5′-CCCCGATAGGTGGACCGCGATTG-3′ and R:5′-CCCGCCCTCTGAACCCCCTACCA-3′) were used to amplify a portion of the *CD40* gene from 100 ng of genomic DNA, 1 × PCR buffer, 200 μm dNTP, 0.1 μm each primers, and 1 U of Taq DNA polymerase (Invitrogen, GM008-1000-5). After amplification, the 10 μl PCR product was digested with *NcoI* enzimi (Fermentas, FD0574) at 37° C 5 min. (digested products 373 bp and 130 bp). After the PCR-RFLP studies of two regions, the digestion products were separated on 1% agarose gels, and fragments stained with the ethidium bromide were photographed on an ultraviolet transilluminator.

Table 1
Baseline clinical and demographical features of the patients with RAS.

Characteristic	Study group, n(%)
Gender, male/female	54/106 (33.8/66.2)
Age, mean ± SD, years	35.69 ± 10,73
Age at disease onset (mean years)	28.18 ± 10.921
Family history of RAS, n (%)	60 (37.5)
Papulopustule, n (%)	17 (10.6)
Systemic involvement, n (%)	1 (0.6)
Erythema nodosum	–
Genital ulcers	–
Pathergy positivity	–

2.3. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 20.0) and the OpenEpi Info software package version 2.2 (www.openepi.com). Results were given as mean ± standard deviation (SD). Chi-square ($\chi 2$) test was used to evaluate the Hardy–Weinberg equilibrium (HWE) for the distribution of the genotypes of the patients and the controls. Chi-square test and Fisher's exact test were used to compare categorical variables appropriately, and odds ratio (OR) and 95% confidence interval (CI) were used for the assessment of risk factors. All p values were 2-tailed, and confidence intervals (CIs) were set at 95%. A p value less than 0.05 was considered as significant.

3. Results

Demographic variables and baseline characteristics of the patients were given in Table 1. Gender, age, age at disease onset, family history, presence of papulopustules and systemic involvement were analyzed in RAS patients. The mean age ± standard deviation (SD) was 35.69 ± 10.73 years in the patients and 37.18 ± 11.91 years in the control group. There were 54 (33.8%) males and 106 (66.2%) females in the patient group. There were not

Table 2
Clinical characteristics of oral ulcers of study patients with RAS.

Characteristic Size, n (%)	Study group n(%)
Minor (MiRAS)	98 (61.3)
Major (MaRAS)	19 (11.9)
Minor + Major	43 (26.9)
Number, n (%)	
1	49 (30.6)
2	73 (45.6)
3	23 (14.4)
≥4	15 (9.4)
Frequency, n (%)	
Once a month	44 (27.5)
Twice a month	30 (18.8)
Three times a month	18 (11.3)
Four times a month	37 (23.1)
Once in 2 months	17 (10.6)
Once in 3 months	14 (8.8)
Period of recovery, n (%)	
1 week	86 (53.8)
2 weeks	66 (41.3)
3 weeks	4 (2.5)
>3 week	4 (2.5)

any patients with erythema nodosum, genital ulcers, and pathergy positivity. No statistically significant association was observed between the clinical and demographic features of RAS patients and *CD40* gene *rs4810485, rs1883832* mutations (Table 1). Table 2 shows the clinical characteristics (size, number, frequency and period of recovery) of oral ulcers of RAS patients. There was also no statistically significant association between characteristics of oral ulcers of RAS patients and *CD40* gene *rs4810485, rs1883832* mutations were observed ($p > 0.05$). Allelic and genotypic distributions of *CD40* gene *rs4810485, rs1883832* mutations in the patients and the controls were shown in Table 3. The observed and expected frequencies of the polymorphism in both patient and control groups were in Hardy–Weinberg equilibrium. It was found that there was no statistically significant difference in the allele and genotype frequencies of both *CD40* gene *rs4810485, rs1883832* mutations between RAS patients and the control groups ($p > 0.05$, OR 0.94, 95% CI 0.70–1.28, OR 1.01 95% CI 0.75–1.37, respectively). Also, no statistical significant difference was detected in the combined genotype analysis of *CD40* gene *rs4810485* and *rs1883832* regions ($p > 0.05$).

4. Discussion

In our study, there was no statistically significant difference between *CD40* gene *rs4810485* and *rs1883832* genotype frequencies of in the patients and the control groups ($p > 0.05$). Additionally, it was found that clinical and demographical characteristics of the patients were not associated with *CD40* gene mutations. Although *CD40*, a member TNF superfamily, is a major contributor of inflammatory processes, it is obvious that *CD40* gene *rs4810485, rs1883832* mutations do not contribute to development of RAS. The results of this study seem to support another study that conducted expression analysis of the *CD40* protein in patients with Behcet's disease, who also have RAS (12). Formation of *CD40-CD40L* complexes also initiates T-cell functions including cytokine secretion, adhesion molecule expression, macrophage and dendritic cell activation, monocyte tumoricidal activity and apoptosis (Subauste, Weesendarp, Sorensen, & Leiva, 1999; Teo & Codarini, 2001; Villarroel et al., 2006). Furthermore, *CD40-CD40L* interactions affects humoral and cellular immunity (Grewal & Flavell, 1996; Villarroel et al., 2006). It was reported that oral ulcers might emerge due to infections which develop as a result of impaired T-cell mediated immunity in hyper-IgM syndrome caused by *CD40L* gene mutations (Teo & Codarini, 2001). Therefore, we think that it is necessary to analyze the gene mutations and expression patterns of *CD40* and *CD40L* in RAS.

Determining the genetic risk factors may uncover the individual susceptibility to RAS. It was suggested that the alterations in the metabolism of cytokines such as pro- and anti-inflammatory interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12), IFN-γ and TNF-α and endothelial nitric oxide synthase genes should be investigated (Slebioda et al., 2013). In the studies that analyzed the *ACE* gene I/D, *MTHFR* C677T and *IL-4* VNTR polymorphisms in patients with RAS, it was found that these gene polymorphisms were positively associated with predisposition to develop RAS (Kalkan, Karakus, & Yigit, 2013a; Kalkan, Yigit, Karakus, Bas, & Seckin, 2013b; Karakus, Yigit, Kalkan, & Sezer, 2013). Also, in another study, it was shown that *IL-1b* and *TNF-α* gene polymorphisms were closely associated with RAS formation (Guimarães et al., 2007). When the studies that aimed to analyze the genetic background of RAS were taken into account, it is seen that they seem to focus on TNF-α and interleukins such as IL-1β, IL-4, IL-6, IL-10 (Bazrafshani et al., 2002; Guimarães et al., 2007; Kalkan et al., 2013b; Slebioda et al., 2013). To date, several polymorphism studies involving various cytokines have been

Table 3
Genotype and allele frequencies of CD40 gene polymorphisms in RAS patient and control groups.

Gene-regions	RAS patients (n:160)	Controls(n:227)	P	OR (CI 95%)
CD40- rs4810485				
Genotypes				
G/G	71 (44.37)	100 (44.05)	>0.05	
G/T	70 (43.75)	106 (46.69)		
T/T	19 (11.87)	21 (9.25)		
Alleles				
G	212 (66.25)	306 (67.40)	>0.05	0.94 (0.70–1.28)
T	108 (33.75)	148 (32.59)		
CD40- *rs1883832*				
Genotypes				
C/C	70 (43.75)	94 (41.40)	>0.05	
C/T	72 (45)	111 (48.89)		
T/T	18 (11.25)	22 (9.69)		
Alleles				
C	212 (66.25)	299 (65.85)		
T	108 (33.75)	155 (34.14)	>0.05	1.01 (0.75–1.37)

performed to investigate the pathogenesis of RAS (Bazrafshani et al., 2002; Borra et al., 2004; Lang et al., 2000; Victoria, Correia-Silva Jde, Pimenta, Kalapothakis, & Gomez, 2005). Although the healthy oral mucosa does not contain many γ, δ T cells, the number of γ, δ T cells increase during development of aphthous. Therefore, it was considered that these cells play a role in the ulceration of oral mucosal (Freysdottir et al., 1999). It is thought that as *CD40* is a member of the TNF superfamily, it could play a role in pathogenesis of autoimmune disease (García-Bermúdez et al., 2012). While *CD40L* is expressed on T cells, *CD40* is expressed on B cells, endothelium and epithelium (Banchereau et al., 1994; Horner, Jabara, Ramesh, & Geha, 1995; Pammer, Weninger, Mazal, Horvat, & Tschachler, 1996). Also, *CD40*, CD44, and CD58 that participated in cell-cell adhesion were moderately expressed by normal oral mucosa (Farmer, Freysdottir, Dalghous, & Fortune, 2001; Kose, Stewart, Waseem, Lalli, & Fortune, 2008). However, it was reported that *CD40*, CD154 and CD80 which are the costimulatory molecules were not normally expressed by the oral mucosal cells (Davies et al., 2012). In the studies that investigated other adhesion molecules, it was reported that the levels of adhesion molecules were elevated during episodes of ulceration. It was also found that there was an association between *E-selectin* rs5361 gene mutation and RAS (Alkhateeb, Karasneh, Abbadi, Hassan, & Thornhill, 2013).

In conclusion, the results of our study suggest that *CD40* gene *rs4810485* and *rs1883832* mutations did not predispose to develop RAS disease in Turkish population. Our findings provide additional information on the possible role of *CD40* in RAS development. The present study is a pioneer research that investigates the association between RAS and the mutations of *CD40* gene, a member TNF superfamily. Therefore, further basic and clinical research with large cohort on different ethnic background is clearly necessary in order to better delineate the complex RAS etiopathogenesis and confirm these findings.

Conflict of interest

None.

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