

Association of *MTHFR* gene C677T mutation with recurrent aphthous stomatitis and number of oral ulcers

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Abstract

Objectives Recurrent aphthous stomatitis (RAS) is a common ulcerative disease of the oral mucosa. Methylenetetrahydrofolate reductase (*MTHFR*) gene variants are associated with thrombophilia and vasculopathy that may result in oral ulceration. Oral ulcers are also the most common feature of Behcet's disease (BD). Association of *MTHFR* gene C677T mutation with BD has been reported in different populations. The aim of the present study was to investigate the possible association between *MTHFR* gene C677T mutation and RAS and evaluate if there was an association with clinical features in a relatively large cohort of Turkish patients. **Materials and methods** The study included 188 patients affected by RAS and 200 healthy controls. Genomic DNA was isolated and genotyped using polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay for the *MTHFR* gene C677T mutation. **Results** The genotype and allele frequencies of C677T mutation showed statistically significant differences between RAS patients and controls ($p=0.002$ and $p=0.0004$, respectively). After stratifying RAS patients according to clinical characteristics of oral ulcers, a significant association was observed between C677T mutation and number of oral ulcers of RAS patients ($p=0.006$).

Conclusions As a result, a high association between *MTHFR* gene C677T mutation and RAS was observed in the present study. Also number of oral ulcers was found to be associated with *MTHFR* C677T mutation in RAS patients.

Clinical Relevance If our observation can be substantiated with further studies, evaluation for *MTHFR* mutations and perhaps folate supplementation may become necessary in selected patients.

Keywords Recurrent aphthous stomatitis (RAS) · *MTHFR* gene · C677T · Genetic susceptibility

Introduction

Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration [1]. Both gender of all ages, races and geographic regions can be affected. It has been estimated that approximately 25 % of the general population might suffer from RAS at some time in their lives [2]. More than 40 % of patients have a familial history of RAS [3].

Recurrent oral ulcers are also one of the common manifestations of Behcet's disease (BD). BD differs from RAS in being a multi-system disease. It was demonstrated that approximately half of all BD patients initially presented with RAS and developed the other features necessary for the diagnosis of BD over the next 7.7 years on average [4]. Association between Methylenetetrahydrofolate reductase (*MTHFR*) gene and BD has been reported in different populations [5, 6].

MTHFR is the enzyme that catalyzes the transformation of homocysteine to methionine via the remethylation pathway (gene located in 1p36) [7]. Hyperhomocysteinemia, a known prothrombotic condition, is the consequence of decreased activity of *MTHFR* [8]. Mutations of the *MTHFR* gene lead to decreased enzymatic activity and may result in a hypercoagulable state. The most common *MTHFR* variant is a point mutation (C→T substitution at nucleotide 677) resulting in an enzyme with 50 % less activity [9]. The C677T mutation of the *MTHFR* gene, causing an amino acid change from alanine to valine, is associated with reduced

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activity and increased thermolability of the enzyme [10]. This mutation is considered the most common genetic cause of elevated homocysteine levels [11, 12]. *MTHFR* mutations and hyperhomocysteinemia may increase the risk of deep-vein thrombosis and arterial occlusive disease [9]. Theoretically, the same mechanism could lead to oral ulceration.

The purpose of the present study was to investigate the *MTHFR* gene C677T mutation in patients with RAS and evaluate if there was an association with clinical features in a relatively large cohort of Turkish patients.

Materials and methods

Subjects

The study population comprised of 188 unrelated patients (66 male and 122 female; mean age: 35.95±11.347 standard deviation [SD] years) with a clinical diagnosis of RAS recruited consecutively and prospectively from those whom were treated and followed up in the Dermatology Department of Gaziosmanpasa University Research Hospital, Tokat, Turkey between January 2012 and December 2012. The diagnosis of RAS was based on accepted clinical criteria [13]. A total of 200 unrelated healthy subjects (76 male and 124 female; mean age: 36.39±12.067 SD years) were recruited consecutively. The control group was comprised of patients with no history of RAS nor systemic diseases, who were admitted to dermatology clinic with other reasons; for nevus examination, treatment of warts, tinea pedis or for contact dermatitis. Exclusion criteria for both groups included the presence of any other significant local or systemic diseases; Behçet's disease, coeliac disease and other gastrointestinal symptoms and/or diseases. HIV testing was performed for all the patients with RAS and all of them had negative HIV

test results. All participants, patients and healthy controls, were of Turkish origin, from the central region of Turkey. The healthy controls matched for age and geographic area with RAS patients. The study protocol was approved by the Local Ethics Committee of Gaziosmanpasa University, Faculty of Medicine, and written informed consent was obtained from the study participants or from the parents when subjects were younger than 18 years.

Genotyping

Genomic DNA was extracted from whole venous blood samples using a commercial DNA isolation kit (Sigma-Aldrich, Taufkirchen, Germany). The *MTHFR* C677T (rs1801133) mutation was analyzed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay as described previously [8]. The amplification conditions consisted of an initial melting step of 5 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 61 °C, and 30 s at 72 °C; and a final elongation step of 5 min at 72 °C. The sequences of PCR primers were 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and 5'-AGG ACG GTG CGG TGA GAG TG-3'. PCR was carried out in a total volume of 25 µl reaction containing 100 ng of genomic DNA, 2.5 µl of 10× PCR buffer, 200 µM dNTP, 10 pM each primers, and one unit of *Taq* DNA polymerase. After amplification, the 198-bp PCR product was digested with *HinfI* in a 15-µl reaction solution containing 10 µl of PCR product, 1.5 µl of 10× buffer, and two units of *HinfI* at 37 °C overnight. The digestion products were separated on 3 % agarose gels, and fragments stained with the ethidium bromide were photographed on an ultraviolet transilluminator. Wild type (CC) individuals were identified by only a 198-bp fragment, heterozygotes (CT) by both the 175/23 and 198 bp, and homozygote variants (TT) by the 175/23 bp.

Table 1 Baseline clinical and demographics features of the 188 study patients with RAS

Characteristic	Total (n=188)	CC (n=102)	CT (n=72)	TT (n=14)	p value
Gender, no. male/female (%)	66/122 (35.1/64.9)	32/70 (31.4/68.6)	29/43 (40.3/64.3)	5/9 (35.7/64.3)	0.479
Age (years)	35.95±11.347	36.69±11.043	35.11±12.388	34.93±7.580	0.629
Age at disease onset (years)	28.24±11.461	27.93±11.034	28.26±12.545	30.36±8.863	0.761
Family history of RAS, n (%)	71 (37.8)	43 (60.6)	26 (36.6)	2 (2.8)	0.122
Papulopustule, n (%)	19 (10.1)	9 (47.6)	9 (47.6)	1 (5.3)	0.679
Systemic involvement, n (%)	1 (0.5)	1 (100)	–	–	0.655
Colchicine use, n (%)	24/167 (14.4)	14 (58.3)	8 (33.3)	2 (8.3)	0.891
Erythema nodosum	–	–	–	–	–
Genital ulcers	–	–	–	–	–
Pathergy positivity	–	–	–	–	–

Mean±standard deviation values are presented for age and age at disease onset

RAS recurrent aphthous stomatitis

Table 2 Clinical characteristics of oral ulcers of 188 study patients with RAS

Characteristics	Total (n=188)	CC (n=102)	CT (n=72)	TT (n=14)	p value
Size, n (%)					0.135
Minor (MiRAS)	113 (60.1)	56 (49.6)	45 (39.8)	12 (10.6)	
Major (MaRAS)	26 (13.8)	14 (53.8)	12 (46.2)	0	
Minor+Major	49 (26.1)	32 (65.3)	15 (30.6)	2 (4.1)	
Number, n (%)					0.006
One	54 (28.7)	28 (51.9)	21 (38.9)	5 (9.3)	
Two	88 (46.8)	58 (65.9)	27 (30.7)	3 (3.4)	
Three	26 (13.8)	10 (38.5)	11 (42.3)	5 (19.2)	
Four or more	20 (10.6)	6 (30.0)	13 (65.0)	1 (5.0)	
Frequency, n (%)					0.471
Once a month	51 (27.1)	29 (56.9)	17 (33.3)	5 (9.8)	
Twice a month	35 (18.6)	19 (54.3)	12 (34.3)	4 (11.4)	
Three times a month	17 (9.0)	8 (47.1)	6 (35.3)	3 (17.6)	
Four times a month	46 (24.5)	22 (47.8)	22 (47.8)	2 (4.3)	
Once in 2 months	22 (11.7)	14 (63.6)	8 (36.4)	0	
Once in 3 months	17 (9.0)	10 (58.8)	7 (41.2)	0	
Period of recovery, n (%)					0.655
One week	96 (51.1)	53 (55.2)	36 (37.5)	7 (7.3)	
Two weeks	84 (44.7)	44 (52.4)	34 (40.5)	6 (7.1)	
Three weeks	4 (2.1)	3 (75.0)	0	1 (25.0)	
>Three weeks	4 (2.1)	2 (50.0)	2 (50.0)	0	

Statistically significant results are shown in boldface
RAS recurrent aphthous stomatitis

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 13.0) and the OpenEpi Info software package version 2.3.1 (www.openepi.com). Results were given as mean±standard deviation (SD). The chi-square (χ^2) test was used to evaluate the Hardy–Weinberg equilibrium for the distribution of the genotypes of the patients and the controls. The relationships between C677T mutation and the clinical and demographics features of patients were analyzed by using χ^2 test or analysis of variance (ANOVA) statistics. χ^2 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 two-tailed, and p values less than 0.05 were considered as significant.

Results

The baseline clinical and demographics features of the study patients with RAS were shown in Table 1. Gender, age, age at disease onset, family history, papulopustule, systemic involvement and colchicine use of RAS patients were analyzed. There was not any patient with erythema nodosum, genital ulcers and pathergy positivity. No statistically significant

Table 3 Genotype and allele frequencies of MTHFR gene C677T mutation in patient and control groups

MTHFR (C677T)	RAS patients n=188 (%)	Healthy controls n=200 (%)	p	OR (CI 95 %)
Genotypes				
CC	102 (54.3 %)	139 (69.5 %)	0.002	
CT	72 (38.3 %)	57 (28.5 %)		
TT	14 (7.4 %)	4 (2.0 %)		
CC+CT : TT	174 : 14	196 : 4	0.011	3.93 (1.33–14.1)
CC : CT+TT	102 : 86	139 : 61	0.002	1.92 (1.27–2.92)
Alleles				
C	276 (73.4 %)	335 (83.8 %)	0.0004	1.87 (1.32–2.66)
T	100 (26.6 %)	65 (16.2 %)		

Statistically significant results are shown in boldface

association was observed between clinical and demographic features of RAS patients and *MTHFR* gene C677T mutation (Table 1). Table 2 shows the clinical characteristics (size, number, frequency and period of recovery) of oral ulcers of RAS patients. There was a statistically significant association between number of oral ulcers and *MTHFR* C677T mutation in RAS patients ($p=0.006$). The frequency of CT+TT genotypes were higher in patients with three and four or more oral ulcers (61.5 % and 70.0 %, respectively) than the patients with one and two oral ulcers (48.2 % and 34.1 %, respectively). Allelic and genotypic distributions of the *MTHFR* gene C677T mutation in patients and controls were shown in Table 3. The observed and expected frequencies of the mutation in both patient and control group were in Hardy–Weinberg equilibrium. The genotype and allele frequencies of C677T mutation showed statistically significant differences between RAS patients and controls ($p=0.002$ and $p=0.0004$; OR=1.9, 95 % CI 1.32–2.66, respectively).

Discussion

RAS is a common disease with oral ulcers. Some causes of ulcerations are vasculopathy, vasculitis, venous stasis and collagen vascular disease. Deep venous thrombosis and arterial occlusive disease risk may be increased in patients with mutations in homocysteine metabolism [9]. *MTHFR* is a key enzyme in folic acid and homocysteine metabolism. This enzyme catalyzes the transformation of homocysteine to methionine. In *MTHFR* gene C677T mutation, C allele is substituted for T allele at nucleotide 677 in the coding region, converting the codon for alanine to valine. Therefore, the final protein has decreased enzyme activity and, consequently, patients develop a mild or moderate hyperhomocysteinemia [14–16]. Both hyperhomocysteinemia and the *MTHFR* gene C677T mutation have been associated with an increased risk of venous and arterial thrombosis in different organs [15, 16]. The mechanism of the effect of homocysteine on coagulation is not completely understood, but in vitro studies have shown that it interferes with the fibrinolytic and anticoagulant system and may damage endothelial cells [15].

To our knowledge, this is the first study to investigate *MTHFR* gene C677T mutation in patients with RAS and it demonstrates a high association between C677T mutation and RAS and number of oral ulcers in RAS patients. Recurrent oral ulcers are also the presenting feature in most cases of BD [17, 18]. Association between *MTHFR* gene C677T mutation and BD and other diseases with oral ulcers, like celiac disease, inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis (UC) and familial Mediterranean fever (FMF), were shown [5, 6, 19–21].

In a recent report, two patients with cutaneous ulcerations, who had heterozygous *MTHFR* mutations, were described

[22]. Both of these patients had dramatic clinical improvement following initiation of B vitamin treatment. Supplementation with folate, vitamin B₁₂ and B₆, which are crucially involved in homocysteine metabolism, reduces homocysteine levels [9, 23]. Several studies have investigated the significance of hyperhomocysteinemia in the context of nutritional alterations in IBD patients and most reports have linked hyperhomocysteinemia to a low folate or vitamin B₁₂ status [24, 25]. It can also be acceptable for RAS because the similarities of these two diseases. It has been demonstrated that individuals with thermolabile *MTHFR* may have a higher folate requirement for regulation of plasma homocysteine concentrations [26].

The exact pathologic link between vascular occlusion and homocysteine metabolism is unclear and maybe due to direct damage to the endothelium, proliferation of smooth muscle within the vessel increases in clotting factors, or decreases in antithrombotic factors. Our report adds to the growing amount of literature raising the questions and prompting further investigation to determine if *MTHFR* mutations and/or hyperhomocysteinemia contribute to the pathogenesis of vascular occlusion. It also suggests that vitamin supplementation, a relatively benign intervention, may improve cutaneous ulcerations associated with altered homocysteine metabolism.

The limitation of the present study is the absence of homocysteine levels of patients and controls. It would be better to measure homocysteine levels of all subjects in order to see the effect of this mutation on homocysteine levels in our study group. Because C677T mutation of *MTHFR* gene is considered the most common genetic cause of elevated homocysteine levels [11, 12], we discussed our results according to this information.

In conclusion, for the first time, our findings demonstrated increased frequency of the TT variant form of C677T mutation in RAS patients. C677T mutation was also associated with number of oral ulcers in these patients. Our findings provide additional support to a genetic basis for RAS development. Further studies are necessary to delineate the complex RAS pathophysiology. Nevertheless, the significance of folate and vitamin B₁₂ deficiency and of hyperhomocysteinemia in cutaneous ulcerations and the impact of their normalization on disease activity require interventional studies. Last but not least, if our observation can be substantiated with further studies, evaluation for *MTHFR* mutations and perhaps folate supplementation may become necessary in selected patients.

Conflicts of interest The authors declare that they have no conflicts of interests.

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