



VEGF, sVEGFR-1 and Endostatin Serum Levels and VEGF Polymorphisms in Recurrent Aphthous Stomatitis

Rekürren Aftöz Stomatitte VEGF, sVEGFR-1 ve Endostatin Serum Düzeyleri ile VEGF Polimorfizmleri

VEGF, sVEGFR-1 and Endostatin in RAS

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Özet

Amaç: Rekürren aftöz stomatit (RAS) oral mukozanın en sık görülen hastalıklarından biridir ve kronik, ağrılı, tekrarlayan ve nekrotizan ülserasyonlarla karakterizedir. RAS'ın kesin etiolojisi ve patogenezini açıklamamıştır. Bu yüzden, Türk RAS'lı hastalarda serum VEGF, sVEGFR-1 ve endostatin düzeylerini; ve ayrıca VEGF +936 C/T ve -1154 G/A tek nükleotid polimorfizmlerinin (SNP'ler) frekanslarını araştırmayı amaçladık. **Gereç ve Yöntem:** 42 RAS'lı hasta (24 minör RAS ve 18 majör RAS) ve 37 sağlıklı birey çalışmaya dahil edildi. Serum VEGF, sVEGFR-1 ve endostatin düzeyleri ELİZA yöntemiyle ölçüldü. VEGF +936 C/T ve -1154 G/A SNP'leri PCR-RFLP metoduyla tanımlandı. **Bulgular:** VEGF serum düzeyi ortalaması +936 C/T SNP'sinin CC genotipini taşıyanlarda CT genotipini taşıyanlara göre daha yüksek bulundu (639.5 ± 309.1 vs 442.1 ± 197.8 ; $p = 0.032$). VEGF -1154 GA ve GG genotipleri sırasıyla minor RAS ve major RAS'lı hastalarda daha sık olarak bulundu ($p = 0.022$). Minor RAS ile major arasında serum VEGF düzeyi ortalaması açısından anlamlı bir farklılık vardı. (677.1 ± 316.7 vs 492.9 ± 242.7 ; $p = 0.032$). **Tartışma:** +936 C/T SNP'sinin T alleli, azalmış serum VEGF düzeyi ile ilişkilidir ve bu azalma, RAS etiolojisinde ve patogenezindeki bozulmuş neovaskülarizasyon ve reepitelizasyon üzerinde yardımcı bir role sahip olabilir. VEGF'nin RAS yatkinliği ve onun klinik göstergelerindeki rolünü belirlemek için daha fazla çalışmaya ihtiyaç duyulmaktadır.

Anahtar Kelimeler

Rekürren Aftöz Stomatit; VEGF; VEGFR-1; Endostatin; Polimorfizm

Abstract

Aim: Recurrent aphthous stomatitis (RAS) is one of the most frequent diseases of the oral mucosa, characterized by chronic, painful, recurrent, and necrotizing ulcerations. The precise etiology and pathogenesis of RAS have not been clarified. Therefore, we aimed to investigate serum levels of VEGF, sVEGFR-1, and endostatin as well as the frequencies of VEGF +936 C/T and -1154 G/A single nucleotide polymorphisms (SNPs) in Turkish patients with recurrent aphthous stomatitis. **Material and Method:** Forty-two patients with RAS (24 minor RAS and 18 major RAS) and 37 healthy subjects were included in the study. Serum levels of VEGF, sVEGFR-1, and endostatin were measured using the ELISA method. VEGF +936 C/T and -1154 G/A SNPs were determined by the PCR-RFLP method. **Results:** The mean serum level of VEGF was found higher in bearing CC genotype of +936 C/T SNP compared with CT genotype (639.5 ± 309.1 vs 442.1 ± 197.8 ; $p = 0.032$). VEGF -1154 GA genotype was found to be more frequent in patients with minor RAS and GG genotype was more frequent in patients with major RAS ($p = 0.022$). There was a significant difference between minor RAS and major RAS with regard to mean VEGF serum levels (677.1 ± 316.7 vs 492.9 ± 242.7 ; $p = 0.032$). **Discussion:** The T allele of +936 C/T SNP is associated with decreased serum VEGF level, and this decrease may have a contributory role in impaired neovascularization and re-epithelialization in the etiology and pathogenesis of RAS. Further studies are needed to determine the role of VEGF in RAS susceptibility and its clinical manifestations.

Keywords

Recurrent Aphthous Stomatitis; VEGF; VEGFR-1; Endostatin; Polymorphism

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Introduction

Recurrent aphthous stomatitis (RAS) is one of the most frequent diseases of the oral mucosa, characterized by chronic, painful, recurrent, and necrotizing ulcerations. These superficial and rounded ulcers can be basically defined as an inflammation containing non-keratinized mucosa. RAS lesions have been classified as minor, major, and herpetiform ulcerations [1]. The precise etiology and pathogenesis of RAS have not been clarified. Several factors including nutrition, food hypersensitivity, drugs, infection, tobacco, hormones, trauma, and psychological stress are generally considered important etiologic factors for the development of RAS [2]. RAS must be distinguished from other diseases which cause recurrent oral ulcers, such as Behcet's disease (BD), celiac disease, Crohn's disease, and systemic lupus erythematosus [3].

Vascular endothelial growth factor (VEGF), a member of a multifunctional growth factor family, has an important role including migration, proliferation, and differentiation of endothelial cells [4, 5]. VEGF is important and necessary both during development and vasculogenesis. VEGF is also considered a pivotal mediator of angiogenesis during wound healing [6, 7]. VEGF has a pleiotropic role in tissue repair via neovascularization, re-epithelialization, and the regulation of extracellular matrix [8]. The gene encoding human VEGF is located on chromosome 6p21.3 and the gene consists of 8 exons [9]. Several single nucleotide polymorphisms (SNPs) have been identified in the VEGF gene [10]. It has been found that the serum VEGF level is increased in patients with BD [11]. The +936 C/T (rs3025039) SNP is located in the 3' UTR of the VEGF gene. Several researchers found that carriers of a +936 T allele had significantly lower VEGF plasma levels than non-carriers [12-14]. The -1154 G/A (rs1570360) SNP is in the promoter region of the VEGF gene. It has been reported that VEGF -1154 A/A and G/A genotypes were linked with low VEGF expression [15-16].

To date, five VEGF ligands have been identified. These ligands bind to three VEGF receptors (VEGFR-1, VEGFR-2, VEGFR-3) as well as to co-receptors, such as neuropilins and heparan sulphate proteoglycans. The VEGFRs induce cellular processes including cell migration, proliferation, and survival. VEGFR-1 acts as a positive regulator for monocyte and macrophage migration, and a positive and negative regulator of VEGFR-2 signaling capacity [17].

Under normal conditions, there is a balance between endogenous angiogenic inducers and endogenous angiogenic inhibitors that keep the angiogenic process in check and prevent inappropriate vascularization of tissues. Angiogenesis inhibitors are often derived from circulating extracellular matrix proteins. Endostatin is a cleaved product of the carboxyl-terminal domain of collagen XVIII [18] and is an endogenous inhibitor of angiogenesis; it may interfere with the pro-angiogenic action of growth factors including VEGF. In vivo and in vitro, endostatin inhibits the migration of endothelial cells and induces apoptosis of endothelial cells [19].

To the best of our knowledge, there is no research investigating the association of VEGF +936 C/T and -1154 G/A SNPs with RAS in the literature. Therefore, in this study, we aimed to investigate serum levels of VEGF, sVEGFR-1, and endostatin as well as the frequencies of VEGF +936 C/T and -1154 G/A SNPs

in Turkish patients with RAS.

Material and Method

Subjects

A total of 42 patients with RAS (18 males, 24 females; mean age: 40.26 ± 14.75 years, range: 15-82 years) and 37 healthy control subjects (19 females, 18 males; mean age: 35.43 ± 18.97 years; range: 17-82 years) were included in the study. All subjects were Caucasian Turkish. Both the patient and control groups were from the same geographical region. The diagnosis of RAS had been made based on accepted clinical criteria [20]. The study was approved by the local ethics committee. All participants were given a written, informed consent.

Measurement of Serum levels of VEGF, sVEGFR-1, and Endostatin

Blood samples of 5 cc were collected from patient and control groups. The samples were centrifuged at 3000 rpm for 10 min and obtained serums were portioned and kept at -80 °C until the analysis time. Then these samples were cooled to room temperature at the same time and serum VEGF (AviBion Human VEGF ELISA Kit, Vantaa, Finland), sVEGFR-1 (Human sVEGFR1/sFLT1 PicoKine ELISA Kit, Boster Biological Technology, Pleasanton, CA, USA), and endostatin (Human Endostatin ELISA Kit, Assay Biotech, Sunnyvale, CA, USA) levels were measured using the enzyme-linked immunosorbent assay (ELISA) method.

Genotyping

Blood samples of 2-3 cc obtained from both RAS patients and control subjects were collected in tubes containing K3EDTA and were stored at -20 °C until the study time. Total genomic DNA was isolated using a commercial DNA isolation kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany). For genotyping of the VEGF SNPs, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used.

VEGF gene +936 C/T and -1154 G/A SNPs were amplified by PCR. The primers for +936 C/T and -1154 G/A were as following: forward: 5'-TCCTGCTCCCTCCTCGCCAATG-3' and reverse 5'-GGCGGGGACAGGCGAGCCTC-3'; and forward: 5'-AGGGTTTC-GGGAACCAGATC-3' and reverse: 5'-CTCGGTGATTTAGAGCAAG-3' respectively. PCR was performed in a final volume of 25 µl using 12, 5 µl 2X PCR master mix (Thermo Fisher Scientific, Massachusetts, USA), 1 µl of each primer and 150-200 ng genomic DNA. After the initial denaturation step at 95°C for 5 min, 35 cycles consisted of denaturation at 95°C for 45 sec, annealing at 62°C for 45 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 10 min. Genotypes were identified by RFLP. Fast digest restriction enzymes, MnlI and NlaIII (Thermo Fisher Scientific, Massachusetts, USA) were used to detect +936 C/T and -1154 G/A SNPs, respectively. PCR products were digested with 1 U of restriction enzymes at 37°C for 5 to 15 min, and then electrophoresis was performed using a 3% agarose gel.

Statistical Analysis

SPSS version 22.0 (SPSS IBM, Armonk, NY, USA) was used for all statistical analyses. For the comparison of independent samples, Student's T-test or Mann-Whitney U test were used.

According to power and sample size analysis, approximately 40 subjects were included in each group to have statistical power of 80%, for a Student's t-test comparing means between the patient and control groups. Chi-square test or Fisher's exact test was used for the comparison of categorical data between RAS patients and controls. One-way ANOVA with Tukey test were used to compare mean serum VEGF levels with regard to genotypes of VEGF -1154 G/A SNP. A p value of less than 0.05 was accepted as significant and all results were given with a 95% confidence interval.

Results

The mean age was 40.26 ± 14.75 years in the patient group and was 35.43 ± 18.97 years in the control group. In terms of age, the difference between the two groups was not significant ($p > 0.05$). Of those in the patient group, 18 were males (42.9%) and 24 were females (57.1%) while the control group was composed of 18 females (48.6%) and 19 males (51.4%). The difference was not significant when the groups were compared in terms of gender ($p > 0.05$). In the study group, there were 24 (57.1%) minor aphthous ulcers and 18 (42.9%) major aphthous ulcers. Recurrence frequencies of oral aphthous ulcer were 30 days in 19 individuals (45.2%), 30-90 days in 17 individuals (40.5%), and 90+ days in 6 individuals (14.3%). Of those in the patient group, 7 (16.7%) had a comorbidity (heart disease, diabetes mellitus, hypertension, etc.).

The number of smokers was 3 and ex-smokers 7 in the patient group: there were no smokers or ex-smokers in the control group. While there was a triggering factor (chocolate, spice, stress, dust mites) in 16 (38.1%) of those in the patient group, there was no triggering factor in the control group. In the patient group, septal deviation was observed in 12 (28.6%) patients while none of the patients in the control group had septal deviation. When the groups were compared for oral hygiene, the difference was not significant ($p > 0.05$). Ten (23.8%) of those in the patient group and 5 (13.5%) of those in the control group were receiving medication (for disease treatment). In the patient group, 30 (71.4%) patients recovered over the short term while 12 (28.6%) patients recovered over the long term.

Table 1 shows the comparison of serum levels of VEGF, sVEGFR-1 and endostatin among RAS, minor RAS, major RAS, and control groups. Compared to controls, mean serum levels of VEGF, sVEGFR-1, and endostatin were not significantly different in patients ($p > 0.05$ for all comparisons). On the other hand, when we compared minor RAS with major RAS, statistically significant difference was found between these two groups with regard to mean VEGF serum levels ($p = 0.032$). Contrary to this,

no significant difference was found with regard to sVEGFR-1 and endostatin serum levels ($p > 0.05$).

When the VEGF +936 C/T and VEGF -1154 G/A gene SNP of the patients and control groups were compared with regard to genotype and allele frequencies, the difference was not found

Table 2. Comparison of genotype and allele frequencies of VEGF gene polymorphisms between patient and control groups

Polymorphism	Genotypes and alleles	Patients (n=42)	Controls (n=37)	P	OR (95% CI)
VEGF +936 C/T Genotype	CC	33 (78.6)	29 (78.4)	-	1*
	CT	9 (21.4)	6 (16.2)	>0.05	1.31 (0.41-4.15)
	TT	-	2 (5.7)	-	-
Allele	CC+CT	9 (21.4)	8 (21.9)	>0.05	0.98 (0.33-2.89)
	C	0.894	0.864	-	1
VEGF -1154 G/A Genotype	T	0.106	0.136	0.05	1.3 (0.49-3.4)
	GG	21 (50.0)	15 (40.5)	-	1
	GA	17 (40.5)	18 (48.7)	>0.05	0.67 (0.26-1.72)
	AA	4 (9.5)	4 (10.8)	>0.05	0.71 (0.15-3.31)
Allele	GA+AA	21 (50)	22 (59.5)	>0.05	0.68 (0.27-1.66)
	G	0.702	0.649	-	1
	A	0.298	0.351	>0.05	0.78 (0.4-1.52)

Data were presented as number and %.

* Reference category

statistically significant (Table 2).

We also examined VEGF genotypes according to size of aphthous ulcer, recurrence frequency, comorbidity, recovery time, triggering factor, and septum deviation. VEGF -1154 GA genotype was found to be more prevalent in patients with aphthous ulcers smaller than 5 mm (minor RAS) and the GG genotype was found to be more prevalent in patients with aphthous ulcers greater than 5 mm (major RAS) ($p = 0.022$). Moreover, +936 CC genotype was observed more frequently in those having no triggering factor (88.5% vs. 62.5%) while the CT genotype was found in those having a triggering factor (37.5% vs. 11.5%). However, this difference was not statistically significant ($p > 0.05$). We did not find any significant association with the other clinical characteristics of patients with RAS (Table 3). Table 4 demonstrates VEGF serum levels with regard to genotypes of VEGF SNPs in RAS, minor RAS, and major RAS. Mean serum level of VEGF was found higher in CC genotype of +936 C/T SNP compared with CT genotype (639.5 ± 309.1 vs 442.1 ± 197.8 ; $p = 0.032$). However, it was not statistically significant in minor and major RAS ($p > 0.05$ for both comparisons). No significant difference was found in terms of mean serum VEGF levels among genotypes of VEGF -1154 G/A SNP ($p > 0.05$ for all comparisons).

Table 1. Comparison of serum levels of VEGF, sVEGFR-1 and endostatin among patient groups and control subjects.

	RAS (n = 42)	Controls (n = 37)	p	Minor RAS (n = 24)	Major RAS (n = 18)	p
VEGF (pg/ml)	596.2 ± 297.9	575.4 ± 315.3	>0.05	677.1 ± 316.7	492.9 ± 242.7	0.032
sVEGFR-1 (pg/ml)	239.9 ± 229.9	243.1 ± 257.6	>0.05	210.5 ± 212.6	279.1 ± 251.8	>0.05
Endostatin (pg/ml)	3782.3 ± 376.4	3759.3 ± 450.7	>0.05	3789.3 ± 397.6	3773.4 ± 358.7	>0.05

Comparisons were performed between RAS and controls, and between minor RAS and major RAS. Data were given as mean \pm SD

Discussion

In the present study, we demonstrated that VEGF +936 C/T and -1154 G/A SNPs and VEGF, sVEGFR-1, and endostatin serum levels were not significantly different in RAS patients compared with controls. In spite of this finding, VEGF -1154 GA genotype was found more prevalent in patients with minor RAS and GG genotype was found more prevalent in patients with major RAS. Moreover, the mean serum level of

Table 3. Comparison of clinical characteristics with regard to VEGF polymorphisms in RAS patients.

Characteristics		VEGF +936 C/T			VEGF -1154 G/A			P
		CC (n=33)	CT (n=9)	P	GG (n=21)	GA (N=17)	AA (n=4)	
Size of aphthous ulcer	Minor RAS < 5 mm	18 (75.0)	6 (25.0)	>0.05	8 (33.3)	14 (58.3)	2 (8.3)	0.022
	Major RAS ≥ 5mm	15 (83.3)	3 (16.7)		13 (72.2)	3 (16.7)	2 (11.1)	
Recurrence frequency	In 30 days	13 (68.4)	6 (31.6)	>0.05	9 (47.4)	8 (42.1)	2 (10.5)	>0.05
	30-90 days	14 (82.4)	3 (17.6)		10 (58.8)	5 (29.4)	2 (11.8)	
	90+ day	6 (100)	-		2 (33.3)	4 (66.7)	-	
Comorbidity	Absent	28 (80.0)	7 (20)	>0.05	19 (54.3)	13 (37.1)	3 (8.6)	>0.05
	Present	5 (71.4)	2 (28.6)		2 (28.6)	4 (57.1)	1 (14.3)	
Recovery time	Short	24 (80.0)	6 (20.0)	>0.05	17 (56.7)	10 (33.3)	3 (10.0)	>0.05
	Long	9 (75.0)	3 (25.0)		4 (33.3)	7 (58.3)	1 (8.3)	
Triggering factor	Absent	23 (88.5)	3 (11.5)	>0.05	15 (57.7)	9 (34.6)	2 (7.7)	>0.05
	Present	10 (62.5)	6 (37.5)		6 (37.5)	8 (50.0)	2 (12.5)	
Septum Deviation	Present	10 (88.3)	2 (16.7)	>0.05	5 (41.7)	6 (50.0)	1 (8.3)	>0.05
	Absent	23 (76.7)	7 (23.3)		16 (53.3)	11 (36.7)	3 (10.0)	

Data were presented as mean ± SD or number and %.

Table 4. VEGF serum levels with regard to genotypes of VEGF polymorphisms in RAS, minor RAS and major RAS.

VEGF (pg/ml)		VEGF +936 C/T		P	VEGF -1154 G/A			P
		CC	CT		GG	GA	AA	
		RAS (n = 42)	(n = 33) 639.5 ± 309.1	(n = 9) 442.1 ± 197.8	0.032	583.5 ± 250.1	606.4 ± 314.5	621.7 ± 517.4
Minor RAS (n = 24)	(n = 18) 735.3 ± 332.0	(n = 6) 512.0 ± 210.3	>0.05	(n = 8) 635.9 ± 258.1	(n = 14) 663.8 ± 314.3	(n = 2) 927.5 ± 642.7	>0.05	
Major RAS (n = 18)	(n = 15) 531.0 ± 248.8	(n = 3) 302.3 ± 43.8	>0.05	(n = 13) 551.3 ± 249.8	(n = 3) 357.6 ± 192.8	(n = 2) 316 ± 127.3	>0.05	

VEGF was found higher in CC genotype of +936 C/T SNP compared with CT genotype in RAS patients. When we compared minor RAS and major RAS, significant difference was found between the two groups with regard to mean VEGF serum levels. Although the etiology of RAS is not fully understood, it is believed to be caused by hereditary factors and changes in the immune reaction against the oral mucosa. A genetic predisposition for the development of aphthous ulcers is strongly suggested to be about 40% for patients with a family history [21,22]. To date, several studies have been reported regarding genetic polymorphisms and RAS susceptibility [23-25].

Saliva has a contributory role in both oral and extra-oral wound healing. It has been known that VEGF is found in human saliva as a part of the major and minor salivary gland secretions. Salivary-derived VEGF also has important roles in mucosal homeostasis [26-28]. However, the role of VEGF in this process is not elucidated. There is a growing body of evidence that vascular endothelium could play a crucial role in recruiting inflammatory infiltrates in RAS [27]. In normal salivary glands, VEGF mRNA and protein are constantly expressed [28]. A stage-dependent fall in salivary levels of VEGF was found in patients with major (but not minor) RAS by Brozovic et al. [29]. Furthermore, decreased salivary VEGF-R3 level was observed in patients with minor RAS [30].

Yalcin et al. [31] showed an increased VEGF expression in correlation with CD34 positivity in oral aphthous of BD. Kamoun et al. [32] have not found any relationship between the VEGF +936 C/T SNP and Behçet's disease susceptibility. However, they found that serum VEGF levels were considerably higher in BD patients. Bozoglu et al. [33] investigated whether VEGF level may be important in the pathogenesis of thrombosis seen in

BD. They found that the levels of VEGF were significantly higher in BD patients with acute thrombosis.

In the current study, we did not find any significant difference between RAS patients and controls with regard to VEGF SNPs and VEGF serum levels. On the other hand, when we compared minor RAS with major RAS, statistically significant difference was found between the two groups with regard to VEGF serum levels. In addition, VEGF -1154 GA genotype was found to be higher in patients with minor RAS and the frequency of GG genotype was higher in patients with major RAS.

Aphthous ulcers are highly angiogenic. Therefore, excess angiogenesis may inhibit re-epithelialization in certain types of ulcers [34]. In a study, lower salivary VEGF levels were found to correlate with impaired neovascularization and re-epithelialization [8]. In the current study, patients bearing CT genotype have been found to have lower VEGF serum levels compared to CC genotype of +936 C/T SNP. We think that the T allele of this SNP may have a contributory role in impaired neovascularization and re-epithelialization.

Hypersensitivity to foods has also been noted as a triggering factor (chocolate, cheese, citrus, tomatoes, seafood, etc.) for RAS development [35]. Allergy or food intolerance is often associated with atopy; a significant association with a history of atopy has been seen in patients with a family history of RAS [36]. The association between RAS and psychological factors including stress and anxiety has been suggested in a growing number of studies [2, 37]. In the current study, while there were not any triggering factor such as chocolate, spice, stress, dust mites in the control group, 16 (38.1%) of those in the RAS group had a triggering factor. Moreover, the CT genotype of +936 C/T SNP was found higher in those having a triggering factor.

The major limitation of the current study was the relatively small number of patients for polymorphism analysis. Thus, in the current study, results of VEGF polymorphisms had low statistical power. Even though polymorphism analysis had a low statistical power, it is the first study of VEGF polymorphisms in RAS patients. We believe that the study has importance because it demonstrates the frequency of VEGF SNPs in RAS patients.

In conclusion, VEGF -1154 GA genotype is more prevalent in patients with minor RAS and GG genotype is more prevalent in patients with major RAS. Moreover, the T allele of +936 C/T SNP decreases serum VEGF level and that decrease may have a contributory role on impaired neovascularization and re-epithelialization in RAS. Further studies are required to determine the role of VEGF in RAS susceptibility and its clinical manifestations.

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Competing interests

The authors declare that they have no competing interests.

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