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Research

Analysis of *Endotheal Nitric Oxide Synthase* Gene VNTR Variant in Turkish FMF Patients

Türk FMF Hastalarında *Endotelyal Nitrik Oksit Sentaz* Geni VNTR Varyantının Analizi

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ABSTRACT

Objective: Familial Mediterranean fever (FMF), caused by the *MEFV* gene encoding pyrin, is a prevalent monogenic autoinflammatory disease. Nitric oxide (NO), synthesized by nitric oxide synthase (NOS) is a gaseous free radical that modulates the immune response. Endothelial NOS *(eNOS)* gene variants may affect NO formation. Therefore, we investigated whether the variable *eNOS* variable number of tandem repeats (VNTR) is involved in the development of FMF. We also examined the association of this variant with clinical findings.

Methods: Three hundred seven subjects, including 147 controls and 160 FMF patients, were genotyped for the *eNOS* VNTR variant using polymerase chain reaction analysis. The patients and controls were compared regarding allele and genotype distribution using the $\chi 2$ test. The results were evaluated statistically.

Results: 51.9% of the patients had two or more MEFV mutations. The most common mutation in the patients was the homozygous M694V/M694V mutation (25%). The genotype and allele frequencies of the *eNOS* gene VNTR variant in FMF patients were all compared with those in the healthy controls. A significant difference was found between the patient and control samples for *eNOS* VNTR genotype distribution. *eNOS* VNTR homozygous 4a/4a and 4b/4b genotypes were higher in patients than those in the controls ($p>0.05$). The patients carrying the 4b/4b genotype had higher colchicine usage and responses to colchicine ($p<0.05$). There was no statistically significant difference between MEFV mutations and *eNOS* VNTR genotype distribution in the patients ($p>0.05$).

Conclusion: This study suggests that the VNTR variant of the *eNOS* gene is associated with FMF formation and some clinical findings in the Turkish population.

Keywords: Familial Mediterranean fever, endothelial nitric oxide synthase, VNTR, polymerase chain reaction

ÖZ

Amaç: Pirini kodlayan *MEFV* geninin neden olduğu Ailesel Akdeniz ateşi (FMF), yaygın tek genli otoenflamatuvar hastalıktır. Nitrik oksit sentaz (NOS) tarafından sentezlenen nitrik oksit (NO) gaz halindeki bir serbest radikaldir ve bağışıklık tepkisini düzenler. Endotelyal NOS *(eNOS)* gen varyantları, NO oluşumunu etkileyebilir. Bu nedenle, *eNOS* değişkeni ardışık tekrar sayısı (VNTR) varyantının FMF gelişiminde yer alıp almadığını araştırmayı amaçladık. Ayrıca bu varyantın klinik bulgularla ilişkisini inceledik.

Gereç ve Yöntem: Yüz altmış FMF hastası ve 147 kontrol dahil olmak üzere 307 kişi, polimeraz zincir reaksiyonu analizi kullanılarak *eNOS* VNTR varyantı için genotiplendirildi. Hastalar ve kontroller, $\chi 2$ testi kullanılarak alel ve genotip dağılımı açısından karşılaştırıldı. Sonuçlar istatistiksel olarak değerlendirildi.

Bulgular: Hastaların %51,9'unda iki veya daha fazla MEFV mutasyonu vardı. Hastalarda en sık görülen mutasyon homozigot M694V/M694V mutasyonuydu (%25). FMF hastalarında *eNOS* geni VNTR varyantının genotip ve alel frekanslarının tümü, sağlıklı kontrollerdekilerle karşılaştırıldı. *eNOS* VNTR genotip dağılımı için hasta ve kontrol örnekleri arasında anlamlı bir fark bulundu. *eNOS* VNTR homozigot 4a/4a ve 4b/4b genotipleri

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hastalarda kontrollere göre daha yüksekti (p>0,05). 4b/4b genotipini taşıyan hastaların kolşisin kullanımı ve kolşisine yanıtları daha yüksekti (p<0,05). Hastalarda MEFV mutasyonları ile *eNOS* VNTR genotip dağılımı arasında istatistiksel olarak anlamlı fark yoktu (p>0,05).

Sonuç: Bu çalışma, *eNOS* gen VNTR varyantının Türk popülasyonunda FMF oluşumu ve bazı klinik bulguları ile ilişkili olduğunu düşündürmektedir.

Anahtar Kelimeler: Ailesel Akdeniz ateşi, endotelyal nitrik oksit sentaz, VNTR, polimeraz zincir reaksiyonu

INTRODUCTION

Familial Mediterranean fever (FMF) is a recessively inherited autosomal inflammatory disease. It particularly affects people in the Mediterranean region, such as Turks, Armenians, Arabs, and non-Ashkenazi Jews. Its characteristics include recurrent episodes of fever, peritonitis, arthritis, rashes, and other serosal inflammations (1). This disease is caused by MEFV mutations, located on the chromosome 16 short arm (2). V726A, M694I, E148Q, M680I, and M694V, mostly located on exon 10, are the mutations of the *MEFV* gene with the highest frequency (3). Mutation of the pyrin protein encoded by the *MEFV* gene has been suggested to cause uncontrolled inflammation.

Nitric oxide (NO), as a gaseous free radical, is synthesized from oxygen and l-arginine and by four main isoforms of NO synthase (NOS), including endothelial NOS *(eNOS)*, neuronal NOS, inducible NOS, and more recently, mitochondrial NOS (4). NO powerfully regulates the immune response, activating a cascade of signal transduction pathways involved in autoimmune and inflammatory responses (5). The *eNOS* gene is located on chromosome 7q35-36. It consists of 26 exons spanning a 21 kb genomic region. Its primary expression is in the low-level endothelial cells in platelets (6). The *eNOS* can stimulate cyclooxygenase 2, KB nuclear factor, and pro-inflammatory cytokines (7). The plasma NO level in healthy people is linked to the variations in the *eNOS* gene. There are different polymorphic sites in the *eNOS*, one of which is VNTR (27 bp repeat, intron 4 VNTR a/b), which causes it to produce basal NO. Due to the possible bonding of the endothelial nuclear proteins to this region, the *eNOS* gene promoter efficiency and then the levels of *eNOS* protein and enzyme activity may be influenced by the VNTR (8). There has been a report of the VNTR five alleles, with 2-6 tandem 27-bp repeats (alleles 2-6) published up to date, among which are alleles 4 and 5 in all populations under study (9). It is shown that the concentration and activity of *eNOS* are lower in the heterozygotes of the 4a/4b variant than those in the homozygotes of 4b/4b (9).

Ongoing chronic inflammation causes endothelial dysfunction. There is a relationship between endothelial dysfunction and the defects in endothelium-based vasodilation mediated by NO (10). It has been reported that endothelial biomarkers are abnormal in patients with FMF.

As the frequency of variants varies between ethnic groups and races, we examined the genotypic and allelic distribution of the *eNOS* VNTR variant in FMF patients in this study. We also evaluated the association between the *eNOS* VNTR variant and MEFV gene mutations.

METHODS

Study Population

The study population included 160 unrelated patients with FMF (89 females and 71 males) who attended the Department of Medical Genetics, Samsun Research and Training Hospital, University of Health Sciences Türkiye, Samsun, Türkiye. Five mutations were examined in patients (M694V, M680I, V726A, E148Q, and A744S). FMF was diagnosed on the basis of the Tel Hashomer criteria. A group of 147 healthy Turkish volunteers (80 females and 67 males) matched by age, sex, and ethnicity with no history or signs of FMF or inflammatory diseases were included as the control group. All the subjects lived in the Central Black Sea region and were over 18 years old. Each patient had detailed clinical characteristics that were recorded. All participants were informed about the study and gave their written informed consent for a protocol. This study was conducted on the basis of the Declaration of Helsinki. The study protocol was approved by the University of Health Sciences Türkiye, Samsun Training and Research Hospital Clinical Research Ethics Committee (protocol no: KAEK 2020/5/13, date: 16.06.2020).

Genotyping

As instructed by the manufacturer, 2-mL blood was taken from each participant, including the controls and the FMF patients, and the commercial kit was used to extract DNA from all samples. *eNOS* VNTR variant was genotyped using the polymerase chain reaction method, which was described previously method (11).

String Analysis

The functional interactions among the proteins are annotated by the STRING database in a cell. In this study, we analyzed the VEGF protein with the STRING database.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows (SPSS, Inc., Chicago, IL) was used

for the analysis of the data. The results are presented as the mean standard deviation (SD). Fisher's Exact test, the χ^2 test, or ANOVA were used to analyze the relationships between the *eNOS* VNTR variant and the patients' demographic and clinical characteristics. Risk factors were assessed using 95% confidence intervals (CIs) and odds ratios (ORs). There were two-tailed p-values, and p-values below 0.05 were regarded as significant.

RESULTS

In this study, 307 subjects, including 147 adult controls and 160 FMF patients, were genotyped for the *eNOS* VNTR variant. The mean age ± SD was 27.23±10.90 in the patients and 37.39±13.39 in the control group. Eighty nine (55.63%) women and 71 (44.37%) men were included in the patient group, and 80 (54.42%) women and 67 (45.58%) men were in the control group. Table 1 shows the patient and control groups' demographic and baseline clinical features.

We evaluated MEFV mutation distributions in FMF patients. 51.9% of patients had two or more MEFV mutations. The most prevalent mutation was the homozygous M694V/M694V mutation (25%). The distribution of MEFV mutations in patients is presented in Table 2.

The genotype and allele frequencies of the *eNOS* gene VNTR variant in FMF patients and healthy controls are given in Table 3. A significant difference was found in the distribution of genotypes of the *eNOS* VNTR variant between patients and controls. *eNOS* VNTR 4a/4a and 4b/4b genotypes were higher in patients compared to controls, respectively [p ($p<0.05$, OR 2.755, 95% CI: 1.380-5.021; $p<0.05$, OR 1.167, 95% CI: 0.928-1.452)]. The patients and the controls did not show significantly different allele frequencies of the *eNOS* VNTR variant ($p>0.05$).

We then evaluated the relationship between the distribution of the MEFV mutations and the genotype distribution of *eNOS* VNTR in the FMF patients (Table 4). No significant difference was found between MEFV mutations and *eNOS* VNTR variants in the patients ($p>0.05$).

We then evaluated the association between the clinical features of FMF and the *eNOS* VNTR genotype distribution (Table 5). Patients with the 4b/4b genotype had higher colchicine usage and responses ($p>0.05$).

When we analyzed the *eNOS* protein with the STRING database, we predicted the protein's functional partners with high confidence (score: 0.7) as follows: ESR1, KDR, VGFA, HSP90AA1, AKT1, CALM1, CAV1, KNG1, NOSTRIN. The interaction network of these proteins is shown in Figure 1.

DISCUSSION

FMF has been classified as a systemic autoinflammatory disorder (12). This disease is common in the Turkish population. Despite the discovery of the responsible gene (*MEFV*), there is no clear link between genotype and phenotype. The *MEFV* gene has a predominant expression in neutrophils (13). Pyrin encoded by MEFV forms the NLRP3 inflammasome complex element and modulates the

Table 1. Baseline clinical and demographics features of the patients with FMF patients and controls

Characteristic	Control group (n=147)	Patient group (n=160)
Gender, male/female, n (%)	67/80 (45.58/54.42)	71/89 (44.37/55.63)
Age, mean ± SD, years	37.39±13.39	27.23±10.90
Diagnose age, mean ± SD, years	-	18.00±12.61
Family history, yes/no, n (%)	-	77/82 (49.4/51.6)
Colchicine usage, yes/no, n (%)	-	91/68 (57.2/42.8)
Response to colchicine, yes/no, n (%)	-	90/69 (56.6/43.4)
Fever status, yes/no, n (%)		143/16 (89.9/10.1)
Abdominal pain, yes/no, n (%)	-	139/20 (87.4/12.6)
Chest pain, yes/no, n (%)	-	49/110 (30.8/69.2)
Joint pain, yes/no, n (%)	-	126/33 (79.2/20.8)
Appendicitis operation, yes/no, n (%)	-	21/138 (13.2/86.8)
Erythema, yes/no, n (%)	-	32/127 (20.1/79.9)
Amyloidosis, yes/no, n (%)	-	13/146 (8.2/91.8)

SD: Standard deviation, FMF: Familial Mediterranean fever

Table 2. Distribution of MEFV gene mutations in FMF patients

MEFV mutations	n (%)
0 mutation	36 (22.5)
1 mutation	42 (26.25)
M694V	22 (13.7)
M680I	14 (8.7)
V726A	2 (1.2)
E148Q	2 (1.2)
A744S	2 (1.2)
≥2 mutations	82 (51.9)
M694V/M694V	40 (25)
M694V/M680I	13 (8.1)
M694V/V726A	3 (1.8)
M694V/E148Q	1 (0.6)
M694V/A744S	1 (0.6)
M680I/M680I	13 (8.1)
M680I/V726A	5 (3.1)
M680I/E148Q	1 (0.6)
V726A/F479L	1 (0.6)
V726A/E148Q	1 (0.6)
E148Q/P369S	2 (1.2)
E148Q/P369S/K695R	1 (0.6)

FMF: Familial Mediterranean fever

pro-inflammatory cytokine interleukin-1 (IL-1β) generation. Therefore, FMF may be regarded as inflammasompathy (14).

Initially, IL-1β is released as a proinflammatory cytokine during attacks, significantly increasing the serum levels of acute-phase reactants such as C-reactive protein (CRP), fibrinogen, erythrocyte sedimentation rates (ESRs), and serum amyloid A (15). The endothelial adhesion molecules, including intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, can facilitate the cellular interactions between B- and T-lymphocytes, monocytes/macrophages, and neutrophils. Endothelial cells can be facilitated by a rich source of proteolytic enzymes and oxygen reactive species, i.e., neutrophils, which are against junctions of the end. This endothelial dysfunction basic mechanism, which neutrophils initiated causes various inflammatory disorders, including FMF (16,17). The pathogenesis of the disease may be caused by several environmental factors and other modifying genes. Studies have found an association between different inflammatory gene variants and FMF (18,19).

Host defense and homeostasis are affected by NO when generated at a low level in the short term; but when it is generated at higher concentrations in the longer term, it becomes mutagenic and genotoxic. For this reason, the NO-mediated effects have complex biological outcomes depending on the external and internal environment of the cell's generation and target sites, as well as the generated

Table 3. Genotype and allele distribution of the eNOS VNTR variant in groups

eNOS VNTR	FMF patients n=160 (%)	Controls n=147 (%)	OR (CI 95%)*	p-value
Genotypes				
4a/4a	9 (5.62)	3 (2.04)	2.755 (1.380-5.021)	**<0.05[&]**
4a/4b	43 (26.87)	59 (40.13)	0.669 (0.463-0.919)	>0.05
4b/4b	108 (67.50)	85 (57.82)	1.167 (0.928-1.452)	**<0.05***
4a4a+4a4b:4b4b	52:108	62:85	0.661(0.41-1.05)	>0.05
4b4b+4a4b:4a4a	9:151	3:144	0.350 (0.07-1.26)	>0.05
Alleles				
4a	61 (19.06)	65	0.830 (0.55-1.23)	>0.05
4b	259 (80.93)	229 (77.89)		
HWE p-value	0.102	0.045		

OR: Odds ratio, CI: Confidence interval, FMF: Familial Mediterranean fever, eNOS: Endothelial nitric oxide synthase, VNTR: Variable number of tandem repeats
*OR (95%CI) corrected according to gender and age, [&]Fisher's Exact test. Statistically significant results were bolded.

NO concentration (20). Different inflammatory diseases such as rheumatoid arthritis (21), osteoarthritis (22), Sjogren's syndrome (23), and systemic lupus erythematosus (SLE) (24) show excessive NO production. Balat et al. (25) reported that total nitrite levels were significantly higher in children with FMF. The endothelial NO production is primarily affected by *eNOS* activity. *eNOS*, which is an enzyme dependent on Ca2+, was first defined in the vascular endothelial cells. *eNOS* function and activity largely affect endothelial function (26). Endothelial dysfunction is heavily affected by oxidative stress caused by *eNOS* dysfunction.

TNF-α, IL-17, cluster of differentiation 40 ligand, interferons, CRP, and SLE-specific circulatory factors have promoted endothelial dysfunction by promoting abnormal *eNOS* function and increasing oxidative stress in recent studies (27). Oxidative stress increased in patients with FMF during attack (28). Yel et al. (29) reported the presence of endothelial damage, particularly during the active disease period, among children with FMF. Additionally, there is little information on endothelial dysfunction and renal involvement in FMF (10). There are two common alleles in the *eNOS* gene 4a/b variant of the 27-bp VNTR in intron 4: 4b with 5 repeats and 4a with 4 repeats (30). The *eNOS* VNTR variant has been suggested to regulate *eNOS* expression by forming small RNAs (siRNAs). There are lower levels of

Table 4. Relationship between MEFV mutations and eNOS VNTR variants in patients

MEFV mutations	eNOS variants	n (%)	p-value
0 mutation	4b/4b	23 (14.4)	
1 mutation	4b/4b	28 (17.5)	
≥2 mutation	4b/4b	57 (35.6)	
0 mutation	4a/4b	8 (5.0)	
1 mutation	4a/4b	12 (7.5)	>0.05
≥2 mutation	4a/4b	23 (14.4)	
0 mutation	4a/4a	4 (2.5)	
1 mutation	4a/4a	2 (1.2)	
≥2 mutation	4a/4a	3 (1.9)	

eNOS: Endothelial nitric oxide synthase, VNTR: Variable number of tandem repeats

Table 5. Evaluation of clinical characteristics according to genotypes in FMF patients

Clinical characteristics		eNOS genotypes			
		4b/4b n (%)	4a/4b n (%)	4a/4a n (%)	p-value
Family history	Yes	51 (32.1)	21 (13.2)	5 (3.1)	>0.05
	No	57 (35.8)	21 (13.2)	4 (2.5)	
Colchicine usage	Yes	54 (34.0)	29 (18.2)	8 (5.0)	**<0.05**
	No	54 (34.0)	13 (8.2)	1 (0.6)	
Response to colchicine	Yes	53 (33.3)	29 (18.2)	8 (5.0)	**<0.05**
	No	55 (34.6)	13 (8.2)	1 (0.6)	
Fever status	Yes	96 (60.4)	38 (23.9)	9 (5.7)	>0.05
	No	12 (7.5)	4 (2.5)	0 (0.0)	
Abdominal pain	Yes	94 (67.9)	37 (23.3)	8 (5.0)	>0.05
	No	14 (8.8)	5 (3.1)	1 (0.6)	
Chest pain	Yes	32 (20.1)	13 (8.2)	4 (2.5)	>0.05
	No	76 (47.8)	29 (18.2)	5 (3.1)	
Joint pain	Yes	83 (52.2)	36 (22.6)	7 (4.4)	>0.05
	No	25 (15.7)	6 (3.8)	2 (1.3)	
Erythema	Yes	26 (16.4)	5 (3.1)	1 (0.6)	>0.05
	No	82 (51.6)	37 (23.3)	8 (5.0)	
Amyloidosis	Yes	9 (5.7)	4 (2.5)	0 (0.0)	>0.05
	No	99 (62.3)	38 (23.9)	9 (5.7)	

FMF: Familial Mediterranean fever, eNOS: Endothelial nitric oxide synthase
*Statistically significant results were bolded.

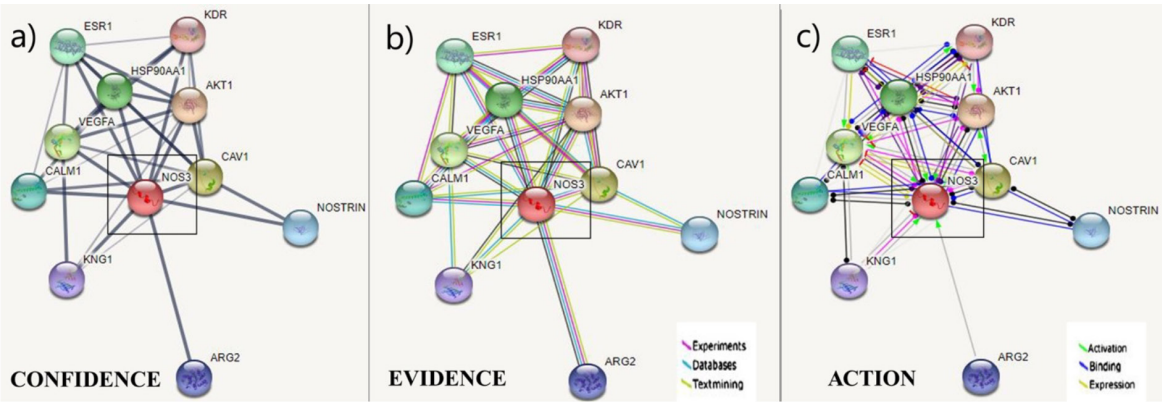

Figure 1. Interactions of Enos protein, according to STRING database predictions: a) confidence network: stronger associations are represented by thicker lines; b) this network represents the types of evidence for the association; c) presentation of the different modes of action, involved in the protein–protein interactions. eNOS protein is evidenced with a square

eNOS and higher quantities of siRNA in the endothelial cells containing five copies than in the cells containing four copies (31). The *eNOS* VNTR variant has been linked to many inflammatory diseases, including autoimmunity diseases such as SLE (32) and Behçet's disease (33).

In this study, we evaluated the distribution of the *eNOS* VNTR variant among Turkish FMF patients and whether this variant is a risk factor for developing FMF. As far as we know, this is the first study on the prevalence of the *eNOS* VNTR variant among Turkish patients with FMF. There was a statistical difference between FMF patients and controls in terms of *eNOS* VNTR variant genotype distribution (Table 2). *eNOS* VNTR homozygous genotypes (4a/4a and 4b/4b) were more common in patients than in controls. When we compared clinical findings with *eNOS* VNTR genotypes, we found that *eNOS* VNTR genotype distribution was associated with the response to colchicine and colchicine usage (Table 5). The responses to colchicine and colchicine usage were higher in patients with the 4b/4b genotype.

Our study had some limitations. First, we could not evaluate other acute complications of FMF. The other one was that our sample size was small.

CONCLUSION

NO's the ability to regulate immune responses has been remarkable over the last two decades. Every cell type in the body virtually produces NO, which is effective in regulating processes at a larger scale, such as the immune and nervous systems. The central issue in FMF is the dysfunction of the innate immune system as a self-reactive autoinflammatory disease. Despite intense research on the pathogenesis of FMF, there are still unknowns. Our results show that the *eNOS* VNTR variant is a risk factor for FMF susceptibility.

ETHICS

Ethics Committee Approval: The study protocol was approved by the University of Health Sciences Türkiye, Samsun Training and Research Hospital Clinical Research Ethics Committee (protocol no: KAEK 2020/5/13, date: 16.06.2020).

Informed Consent: All participants were informed about the study and gave their written informed consent for a protocol.

Authorship Contributions

Concept: Ö.S.T., S.Y., Design: A.F.N., A.T., Data Collection or Processing: Ö.S.T., Analysis or Interpretation: S.Y., A.T., Literature Search: A.F.N., Writing: A.F.N.

Conflict of Interest: No conflict of interest was declared by the authors.

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