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ASSOCIATING *eNOS* GENE VARIANTS WITH COVID-19 SUSCEPTIBILITY IN THE TURKISH POPULATION

TÜRK POPÜLASYONUNDA COVID-19 DUYARLILIĞI İLE *e*NOS VARYANTLARININ İLİŞKİSİ

Naci ŞENKAL[1], Yasemin OYACI[2], Timurhan CEBECİ[1], Hilal KONYAOĞLU[1], Murat KÖSE[1], Mustafa ÖNEL[3], Alpay MEDETALİBEYOĞLU[1], Gözde YEŞİL SAYIN[4], Mustafa PEHLİVAN[5], Sacide PEHLİVAN[2], Ümmihan İŞOĞLU-ALKAÇ[6], Tufan TÜKEK[1]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine Istanbul, Turkiye
[2]Istanbul University, Istanbul Faculty of Medicine, Department of Medical Biology Istanbul, Turkiye
[3]Istanbul University, Istanbul Faculty of Medicine, Department of Microbiology, Istanbul, Turkiye
[4]Istanbul University, Istanbul Faculty of Medicine, Department of Medical Genetics Istanbul, Turkiye
[5]Gaziantep Univesity, Faculty of Medicine, Department of Haematology, Gaziantep, Turkiye
[6]Istanbul University, Istanbul Faculty of Medicine, Department of Physiology, Istanbul, Turkiye

ORCID IDs of the authors: N.S. 0000-0001-7072-8724; Y.O. 0000-0002-1338-0087; T.C. 0000-0002-0966-2436; H.K. 0000-0003-3036-9143; M.K. 0000-0002-7487-9287; M.Ö. 0000-0002-3987-6611; A.M. 0000-0002-5829-9186; G.Y.S. 0000-0003-1964-6306; M.P. 0000-0002-6692-085X; S.P. 0000-0003-1272-5845; Ü.İ.A. 0000-0003-1992-0109; T.T. 0000-0002-4237-1163

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ABSTRACT

Objective: COVID-19 is a serious respiratory and vascular disease that impairs the protective function of the endothelial barrier. Endothelial nitric oxide synthase (eNOS), the most important isoform for nitric oxide (NO) production, is mostly expressed in endothelial cells. Therefore, this study aims to evaluate whether *eNOS* G894T and variable tandem repeat number (VNTR) functional variants show predisposition to developing COVID-19.

Materials and Methods: The study includes a total of 384 subjects (284 COVID-19 patients and 100 healthy controls). Two *eNOS* gene variants (G894T and VNTR) were genotyped using the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods, with the results being evaluated using statistical methods.

Results: A significant association has been identified between *eNOS* G894T and COVID-19. For the eNOS G894T variant, the T/T genotype (p=0.035) and T allele carriers (p=0.030) appear to have an increased risk of developing COVID-19. The eNOS G894T G/G genotype (p=0.030) was more common in the control group compared to the patient group. No significant difference was found between groups regarding the *eNOS* VNTR genotype and allele frequencies (p>0.05). The genotypes of

ÖZET

Amaç: Ciddi bir solunum ve damar hastalığı olan COVID-19 hastalığında endotel bariyerinin koruyucu işlevi bozulmaktadır. Nitrik oksid (NO) üretimi için en önemli izoform olan endotelyal NO sentaz (eNOS), çoğunlukla endotel hücrelerinde eksprese edilir. Bu nedenle, bu çalışma, eNOS G894T ve değişken ardışık tekrar sayısı (VNTR) fonksiyonel varyantlarının COVID-19 hastalığının gelişimine yatkınlık oluşturup oluşturmadığını değerlendirmeyi amaçladı.

Gereç ve Yöntem: Bu çalışmaya toplam 384 birey (284 COVID-19 hastası ve 100 sağlıklı kontrol) dahil edildi. İki *eNOS* gen varyantı (G894T ve VNTR), polimeraz zincir reaksiyonu (PCR) ve/veya kısıtlama fragman uzunluğu polimorfizmi (RFLP) yöntemleri ile genotiplendi. Sonuçlar istatistiksel yöntemlerle değerlendirildi.

Bulgular: eNOS G894T ile COVID-19 hastalığı arasında anlamlı bir ilişki tespit edildi. eNOS G894T varyantı için, T/T genotipi ve T aleli taşıyıcılarının COVID-19 hastalığı için artan riske sahip olduğu görüldü (sırasıyla, p=0,035 ve p=0,030), eNOS G894T G/G genotipi, kontrol grubunda hasta grubuna göre daha yaygındı (p=0.030). eNOS VNTR genotipi ve allel frekansları açısından gruplar arasında anlamlı farklılık yoktu (p>0.05). Bu varyantlar için

Corresponding author/İletişim kurulacak yazar: Timurhan CEBECİ – timurhancebeci19@gmail.com

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the patient and control groups for these variants were in Hardy-Weinburg equilibrium (HWE).

Conclusion: These results provide evidence supporting the hypothesis that the eNOS G894T variant is associated with an increased risk of developing COVID-19 in the Turkish population. These findings may lead to the emergence of new treatment options. Further research is required to understand the molecular mechanisms involved in the pathogenesis of the disease.

Keywords: COVID-19, nitric oxide synthase, variant, RFLP-PCR

hasta ve kontrol gruplarının genotipleri HWE'deydi.

Sonuç: Bu sonuçlar, eNOS G894T varyantının Türk popülasyonunda artan COVID-19 riski ile ilişkili olduğu hipotezini destekleyen kanıtlar sağlamıştır. Bulgularımız yeni tedavi seçeneklerinin ortaya çıkmasına yol açabilir. Hastalığın patogenezinde yer alan moleküler mekanizmaları anlamak için daha fazla araştırmaya ihtiyaç vardır.

Anahtar Kelimeler: COVID-19, nitrik oksit sentaz, varyant, RFLP-PCR

INTRODUCTION

COVID-19 is caused by the coronavirus 2 (SARS-CoV-2) and is a respiratory and vascular disease with severe symptoms (1). About 50% of COVID-19 patients are asymptomatic or mildly symptomatic. However, 3-10% of patients require hospitalization, up to 20% of which may result in death (2). A COVID-19 infection involves a cytokine storm, in which large amounts of proinflammatory cytokines are released and an aggressive inflammatory response is observed. In this stage, proinflammatory cytokines increase in the circulatory system (3). The clinical picture shows cell death and diffuse endotheliitis directly related to viral infection to occur in patients who eventually die as a result of rapid worsening and multi-organ failure (4). This indicates COVID-19 to impair the protective function of the endothelial barrier, which allows a systemic viral invasion. Furthermore, altered vascular barrier integrity has been linked to pulmonary endothelial cell injury and the initiation and spread of acute respiratory distress syndrome (ARDS), which is the leading cause of death in COVID patients (4, 5). High levels of D-dimers, which are produced from the coagulation and fibrin degradation caused by endothelial cell death, indicate a poor prognosis in COVID-19 patients. Conversely, a COVID infection may end without thrombotic complications in patients with mild symptoms. This suggests the integrity of endothelial cells to be important in preventing the occurrence of COVID-19 (6).

Nitric oxide (NO) is produced from L-arginine through nitric oxide synthase (NOS) and has an important function in vascular tone and blood pressure control, and NOS has three different types: endothelial (*e*NOS), inducible (*i*NOS), and neuronal (*n*NOS) (7), *e*NOS is mostly expressed in endothelial cells. Systemic hypertension, platelet aggregation, and a number of vascular diseases including thrombosis and atherogenesis have been observed in *e*NOS-/- mice (8). The vasodilatory and platelet aggregation-inhibiting properties of eNOS-derived NO appear to act as an antiviral defense mechanism. By inactivating viral replication proteins, NO either directly or indirectly inhibits viral replication and protein production in host cells (9), SARS-CoV being one of these (10). The *eNOS* gene is localized on the human chromosome 7q36, which consists of 27 exons and encodes a protein of 1,203 amino acids. Many variants have been identified in the *eNOS* gene, with the most investigated variants being G894T in exon 7 (rs1799983) and variable tandem repeat number (VNTR) in intron 4 (rs61722009). These variants can alter the expression and activity of the *e*NOS enzyme (11). Therefore, this study aims to evaluate whether *e*NOS G894T and VNTR functional variants show predisposition to COVID-19 development.

MATERIALS AND METHODS

Study population

The study sample includes 234 unrelated PCR-confirmed patients with COVID-19 and 100 healthy controls. The patients with COVID-19 and the controls were recruited from the internal medicine department of a university research hospital in Türkiye. The diagnosis was confirmed by a positive result obtained from the real-time reverse transcriptase polymerase chain reaction (RT-PCR) of oropharyngeal and nasopharyngeal swab samples taken from patients with a suspected infection. The control group was composed of individuals who, alongside their families, have not been exposed to the virus and/or disease and have had a negative RT-qPCR test for over 7 months. All subjects in this study are older than 18 years and are part of the Turkish population from Türkiye's Marmara region. The university clinical research ethics committee approved the protocol of the study (Date: 08.05.2020, no:09), and the study was carried out in accordance with the Declaration of Helsinki.

Genotyping

DNA isolation was performed from the blood taken from the groups in accordance with the manufacturer instructions using a commercial kit. *e*NOS G894T and the VNTR variants were genotyped using the polymerase chain reaction (PCR) and/or restriction fragment length polymorphism (RFLP) methods, as previously described (12). The *e*NOS G894T variant was identified through PCR using the following primers: (F) 5′ CATGAGGCTCAGCCCCAGAAC-3′ and (R) 5′-AGTCAATCCCTTTGGTGCTCAC-3′. The PCR products were digested overnight by the MboI enzyme (Invitrogen, Carlsbad, CA, USA) at 37°C. Next, the fragments were separated on a 2% agarose gel electrophoresis and visualized using ultraviolet light. The 206 bp products had

a consistent restriction site, resulting in a 119 bp and an 87 bp fragment. The *eNOS* VNTR variant was evaluated through PCR amplification using the primers (F) 5'-AGG-CCCTATGGTAGTGCCTTT-3' and (R) 5' TCTCTTAGTGCT-GTGGTCAC-3'. The amplified products were separated by electrophoresis on a 2% agarose gel and visualized using ethidium bromide staining. The wild-type allele had five tandem repeats of 27 and 420 bp, while the mutant allele had four tandem repeats of 27 and 393 bp.

Statistical analysis

Statistical analysis was performed using the program SPSS (version 22.0) for Windows (SPSS Inc., Chicago, IL, USA). The differences between groups were analyzed using logistic regression analysis, with the odds ratio (OR) at a 95% confidence interval (CI) also being calculated. The *e*NOS genotype distribution of groups was compared using the chi-square test, with Fisher's exact test being used as needed. The Hardy-Weinberg equilibrium (HWE) was then calculated, with a *p*-value less than 0.05 being considered statistically significant.

RESULTS

The 334 subjects (234 COVID-19 patients and 100 healthy controls) were evaluated for the *eNOS* G894T and VNTR variants. The distribution of the genotypes and alleles of the *eNOS* G894T and VNTR variants is presented in table 1. The *eNOS* G894T genotype and allele comparison between groups showed a significant difference. For *e*NOS G894T, a higher frequency was observed for the G/T (*p*=0.035; OR=9.469, 95% CI[1.170, 76.603]) and T/T genotypes (*p*=0.013; OR=8.313, 95% CI[1.106, 62.494]) in the patients compared to the controls. The *e*NOS G894T variant G/G genotype was more common in the control group compared to the patient group (*p*=0.030; OR=9.883, 95% CI[1.243, 78.578]).

The prevalence of the genotypes 4b/4b, 4a/4b, and 4a/4a profiles for the *e*NOS VNTR variant were 69%, 28.2%, and 2.8% in patients, and 68%, 28%, and 4% in the control group, respectively. No significant difference was observed between groups regarding the *e*NOS VNTR genotype and allele distribution (p>0.05). The genotypes of both groups regarding the *e*NOS G894T and VNTR were in HWE.

DISCUSSION

COVID-19 has a heterogeneous clinical phenotype, in which severe symptoms such as endothelial inflammation, thromboembolic complications, acute respiratory distress syndrome (ARDS), and multiple organ failure

Table 1. Genotype and allele distribution of *eNOS* gene variants in patients and controls

eNOS G894T	Patients n=284 (%)	Controls n=100 (%)	OR Exp (B)	95% CI	p*
Genotypes		n=100 (%)			
G/G	146 (51.4)	63 (63)	9.883*	1.243-78.578*	**0.030***
G/T	116 (40.8)	36 (36)	9.469*	1.170-76.603*	**0.035***
T/T	22 (7.8)	1 (1)	8.313[&]	1.106-62.494[&]	**0.013[&]**
Alleles					
G	408 (71.8)	164 (81.0)	1.672[&]	1.123-2.489[&]	**0.011[&]**
T	160 (28.2)	38 (19.0)			
HWEp	0.875	0.089			
eNOS VNTR	**Patients n=284 (%)**	**Controls n=100 (%)**	**OR Exp (B)**	**95% CI**	**p***
Genotypes					
4b/4b	196 (69.0)	68 (68.0)	0.696[&]	0.205-23.62[&]	0.519[&]
4b/4a	80 (28.2)	28 (28.0)	1.179*	0.663-2.095*	0.576*
4a/4a	8 (2.8)	4 (4.0)	1.076*	0.293-3.953*	0.912*
Alleles					
4b	472 (83.1)	164 (82.0)	0.927[&]	0.607-1.413[&]	0.744[&]
4a	96 (16.9)	36 (18.0)			
HWEp	0.962	0.606			

HWEp: *Hardy*-Weinberg Equilibrium *:OR (95%CI) adjusted for age and gender, [&]Fisher's Exact Test. Statistically significant results are in bold.

may occur, as well as asymptomatic or mild findings (13). The endothelial cell layer between the blood and tissues forms an anatomical barrier against viruses in the body. The endothelium is found in many organs in the human body and provides vascular homeostasis through the interaction of the vessel walls and cells in the lumen. The endothelium also balances the production of NO, which is the most important compound in vasodilators, and thus regulates vascular tone (14). Permeable viruses such as viremic SARS-CoV-2 (80–100μnm in size) have the ability to invade the local tissue underneath the endothelium, and endothelial dysfunction is usually seen in infections caused by highly pathogenic coronaviruses (15). This feature of SARS-CoV-2 infections damages pulmonary and other vascular endothelia (7). In endothelial dysfunction, the bioavailability of NO generally decreases, while endothelin-1 (ET1), angiotensin II (Ang II), and other similar vasoconstrictor substances increase (16).

NO plays a role in regulating immune responses against pathogens and also regulates the cellular function, growth, and death of various immune cells (17). Although NO has a protective role in viral infections, it may contribute to the molecular mechanism of COVID-19. Pathophysiological conditions that allow NO release to lead to the formation of reactive oxygen species (ROS) (18). Excess ROS produced by endothelial and epithelial cells and leukocytes is important in ARDS progression and lung injury. The majority of vascular NO production is mediated by *e*NOS. The *eNOS* gene is highly polymorphic and has several variants, such as single nucleotide polymorphisms (SNPs), insertions/deletions, VNTRs, and microsatellites (19). The *e*NOS G894T variant resulting from a substitution of thymine for guanine at position 894 in exon 7 of the *eNOS* gene causes a glutamine-aspartate exchange at position 298 of the protein (20). This functional variant leads to posttranslational modifications. The presence of the allele-specific primers (ASP) in this variant reduces *e*NOS levels by reducing the binding of *e*NOS to caveolin-1 (21). This effect results in reduced *e*NOS activity and NO formation (21). *In vitro* studies have reported that carriers of the variant allele reduce platelet NO production compared to those carrying the wild allele. As another variant in the current study, the 4b/4a VNTR variant in intron 4 transcriptionally regulates *e*NOS by altering small interfering RNA (siRNA) formation (22). The most common alleles in this variant are those with five (4b variant) or four (4a variant) copies of the 27-bp DNA fragment (23). *In vitro* studies have indicated the 4b variant to show higher amounts of siRNA in endothelial cells, resulting in lower *e*NOS mRNA levels compared to the 4a variant (24). Various studies are found to have examined the associations these variants have with different diseases, such as essential hypertension, prostate cancer, colorectal cancer, and recurrent spontaneous abortion (25-28). Zhao et al. reported *e*NOS G894T to be related to *Mycoplasma pneumoniae* in Chinese children (29). A meta-analysis study found *e*NOS G894T genotype frequencies and the T allele to differ between high-altitude pulmonary edema patients and controls in Asians (30). A study on enterovirus 71 (EV71) found the T allele of *e*NOS G894T to be related to EV71 infection (31). That same study found low NO levels for people infected with EV71 who have the T allele. The *e*NOS G894T and VNTR genotype and allele distribution have been reported to not differ between sepsis patients and controls in Turkish patients (32). Koskela et al. reported the *e*NOS G894T TT genotype to be associated with the severity of Puumala hantavirus (PUUV) infection, with infected patients possessing the TT homozygous genotype to show more severe clinical presentations and longer hospital stays compared to other genotypes (33). A study comparing severe COVID-19 and mild COVID-19 patients found the distribution of the *e*NOS G894T genotype frequencies to not differ between the two groups (34). A previous study also found the genotype and allele distribution of *e*NOS G894T and VNTR to not different between PCR-negative COVID-19 patients and controls (35).

This study has investigated the association of *eNOS* G894T and intron 4 VNTR variants with the risk of COVID-19 and has found a significant difference to exist between patients and controls regarding *e*NOS G894T, with the *e*NOS G894T TT genotype and T allele being more common in patients compared to controls. The patients carrying the T allele were also found to be predisposed to developing COVID-19. The study has shown the G894T variant in exon 7 to cause a mature NOS protein that is sensitive to intracellular division, which may reduce functional *e*NOS activity in those possessing the T allele (36). The *e*NOS VNTR variant genotype and allele distribution revealed no difference between the patient and control groups.

This study has certain limitations. First, it was carried out only with patients living in a particular area of Türkiye. Different results may be obtained by studying people living in different regions of Türkiye. Second, the study only evaluated two variants in the *eNOS* gene. Other functional variants in the *eNOS* gene may also contribute to disease occurrence. In addition, gene-environment interactions may also need to be evaluated.

In conclusion, having sufficient knowledge about the etiology and pathogenic processes associated with COVID-19 is just as important as knowing the factors that play a role in the disease. This information can contribute to estimating the risk of COVID-19 disease and taking more effective measures. Genetic variants play a role in the susceptibility to many diseases such as cancer, autoimmune diseases, and infections. Studies have shown various genetic variants and environmental factors to influence the course of COVID-19. The findings from this

study support the hypothesis that eNOS G894T is linked to the occurrence of COVID-19. Individuals who carry the eNOS G894T, T/T genotype, and T allele were found to be more likely to develop COVID-19, and this study's findings may lead to the emergence of new treatment options. Further research is required to understand the molecular mechanisms involved in the pathogenesis of the disease.

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