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A Strong Association Between COVID-19 and Single Nucleotide Polymorphisms in Nuclear Factor Kappa B genes

COVID-19 ile Nükleer Faktör Kappa B Genlerindeki Tek Nükleotid Polimorfizmleri Arasındaki Güçlü Bir İlişki

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ABSTRACT

Objectives: SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus belonging to the Betacoronavirus genus. This study aimed to investigate the relationship between COVID-19 severity and NF-κB1 -94 ins/del (rs28362491), NF-κB1A 3'UTR A/G (rs696), -826 C/T (rs2233406) polymorphisms.

Materials and Methods: We investigated the frequencies of these gene polymorphisms in 150 patients with COVID -19 and 171 healthy controls. Total DNA was isolated from the blood samples, and then the PCR-RFLP study was used for genotyping. All statistical analyses were calculated using the chi-square method using SPSS.

Results: A statistically significant differences were determined in the D allele frequency, WD and DD genotype frequencies for the rs28362491 polymorphism. For rs696 polymorphism, there was a statistically significant difference in the frequency of the *G* allele of patients with COVID-19. Additionally, for this polymorphism, CT and TT genotype frequencies were shown to be statistically significant. It was also found that the T allele, CT, and TT genotype frequencies for the rs2233406 have a statistically significant difference.

Conclusion: A significant association was found between COVID-19 disease and NF-κB genes, but further studies, such as investigating promoter activity or gene expression levels, are needed.

Keywords: COVID-19, NF-κB1-94 Ins/Del ATTG, NF-κB1A 3'UTR A/G, NF-κB1A -826 C/T, polymorphism

ÖZ

Amaç: SARS-CoV-2, Betacoronavirus cinsine ait zarflı, pozitif polariteli, tek sarmallı bir RNA virüsüdür. Bu çalışmada COVID-19 hastalığının şiddeti ve NF-κB1 -94 ins/del (rs28362491), NF-κB1A 3'UTR A/G (rs696), NFk-B1A -826 C/T (rs2233406) polimorfizmleri arasındaki ilişkinin araştırılması amaçlanmıştır.

Materyal ve Metot: COVID-19'lu 150 hastada ve kontrol olarak 171 sağlıklı bireyde bu genlerin polimorfizm sıklıklarını araştırdık. Kan örneklerinden total DNA izole edildi ve ardından genotipleme için PCR-RFLP çalışması kullanıldı. Tüm istatistiki analizler SPSS kullanılarak ki-kare yöntemi ile hesaplanmıştır.

Bulgular: Rs28362491 polimorfizmi için D alel frekansı, WD ve DD genotip frekanslarında istatistiksel olarak anlamlı farklılıklar tespit edilmiştir. Rs696 polimorfizmi için, COVID-19'lu hastaların G aleli sıklığında istatistiksel olarak anlamlı bir fark vardı. Ayrıca, bu polimorfizm için CT ve TT genotip frekanslarının istatistiksel olarak anlamlı olduğu gösterilmiştir. Ayrıca, rs2233406 için T aleli, CT ve TT genotip frekanslarının istatistiksel olarak anlamlı bir farka sahip olduğu bulunmuştur.

Sonuç: COVID-19 hastalığı ile NF-κB genleri arasında anlamlı bir ilişki bulunmuştur, ancak promotör aktivitesinin veya gen ekspresyon seviyelerinin araştırılması gibi daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: COVID-19, NF-κB1-94 Ins/Del ATTG, NF-κB1A 3'UTR A/G, NF-κB1A -826 C/T, polimorifzm

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INTRODUCTION

Coronavirus Disease (COVID-19) is an infectious disease caused by the *SARS-CoV-2* coronavirus that emerged in China, firstly in Wuhan, and spread all over Asia.[1] The virus is a positive-polarity, enveloped and single-stranded RNA virus of the genus Betacoronavirus and family coronavirus.[2] Although *SARS-CoV-2* is mainly characteristic of animals, they can infect other species, including humans.[3] Immune response developed against COVID-19 infection is quite similar to other viruses. Immunity is associated with inflammation, and an appropriate and integrated functioning of both processes is essential. Viral particles are defined as foreign elements to the body, as in every infection.[4] This results in the production of proinflammatory factors, including cytokines.[5] Impaired modulation of genes directly involved in inflammation-related processes, including genes encoding cytokines and chemokines, has been shown due to abnormal activation of Nuclear Factor kappa B (NF-κB).[6] NF-κB is also an important regulator of innate immune cells such as T or B cells. Therefore, a dysregulation in NF-κB can lead to an uncontrolled and pathogenic inflammatory response. Interestingly, upregulation of NF-κB has been observed to play a role in the development of *SARS-CoV-2* infection.[6]

The NF-κB1 gene is positioned on chromosome 4q21. The -94 Ins/Del ATTG (rs28362491) polymorphism is a functional polymorphism located in the promoter of this gene. One of the protein families that can manage many inflammatory events is NF-κB. IκBα, encoded by NF-κB1A, is an important inhibitor of NF-κB activity.[7] NF-κB1A genes located on chromosome 14q13 have been associated with the development of many cancers.[8] In addition, -826 C/T polymorphism was associated with hepatitis B virus.[9] Many polymorphisms have been shown in the NF-κBIA gene, but the A/G polymorphism in the 3'UTR region, which is potentially functionally prominent, is thought to regulate gene expression effectively.[10] The 3'UTR A/G polymorphism identified by Glavac et al.[10] and Gao et al.[11] was associated with various cancer types. It is thought that the variation most likely affects the expression of NF-κBIA, which in turn alters the structure and function of the protein, leading to its weak binding to the NF-κB complex and consequently leading to NF-κB activation.

We hypothesize that NF-κB1-94 Ins/Del ATTG, NF-κB1A 3'UTR A/G, and NF-κB1A -826 C/T polymorphisms may act as risk factors for COVID-19 disease. This study aimed to investigate whether functional polymorphisms in NF-κBI and NF-κBIA are associated with COVID-19 disease and its severity.

MATERIALS AND METHODS

Ethics Committee Approval: Approval was obtained from the Sivas Cumhuriyet University Clinical Research Ethics Committee (Decision No: 2021-02/07). The control group consisted of 171 healthy individuals whose blood was drawn during the period when there was no COVID-19 outbreak (Decision No: 2009-02/5). Informed consent forms were obtained from all volunteers.

Collection and storage of the samples: We investigated the genotype and allele frequencies of NF-κB1 -94 Ins/Del ATTG, NF-κB1A 3'UTR A/G, and NF-κB1A -826 C/T polymorphisms in this study. Blood samples of patients diagnosed with COVID-19 were used in this study. The patient group consisted of 150 individuals with a definitive diagnosis of COVID-19 disease by the Sivas Cumhuriyet University Faculty of Medicine Research Hospital Infectious Diseases Department. Individuals who have not been diagnosed with COVID-19 or have any chronic or infectious disease will not be included in the patient group. First, DNA was extracted from blood samples of COVID-19 patients. Polymerase chain reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) was performed for rs28362491, rs696, and rs2233406 genotyping. According to hematologic, biochemical, and serologic laboratory findings, the patients had no other infection or chronic disease. Patients were categorized as severe and non-severe in terms of disease severity. COVID-19 patients hospitalized in the intensive care unit and asymptomatic ones were classified as severe and non-severe, respectively. Healthy volunteers had no disease complaints in their medical history, and their examinations were normal.

Determination of Gene Polymorphisms: Blood samples were stored at -20^0 C until the time of the study. Total genomic DNA isolation was performed using the standard phenol-chloroform protocol.[12] The concentration of DNA was determined in ultraviolet-visible spectroscopy (UV–VIS) nanodrop (Maestro, NANO). PCR-RFLP method was used to determine the genotypes of the individuals. Within the scope of the study, the appropriate primer pairs and annealing temperatures for the relevant gene regions of the genomic DNA isolated from blood samples were determined as in our previous studies.[13,14] PCR products of the relevant gene regions were cut with appropriate restriction enzymes. The digested products were run on a 3% agarose gel, photographed in a gel imaging system, and the genotypes of the individuals were determined (Figure 1). In order to eliminate errors such as partial digestion that may arise from restriction digestion, samples from the patient and control group (15%) with different genotypes were selected, and the genotypes of

the individuals were confirmed by DNA sequence analysis.

Statistical Analysis: Statistical analysis was formed by comparing patients with COVID-19 in intensive care with individuals with moderate and mild disease severity. Data were uploaded to the SPSS (Ver: 23.0) program, and Pearson's chi-squared test calculated the NF-κB genes alleles and genotype frequencies between case and control groups. Descriptive statics are presented as percentages and frequencies for categorical variables and as medians for continuous variables. Analysis of haplotype frequencies was performed using SHEsis online software (http://analysis.bio-x.cn/myAnalysis.php) and this software was used for possible haplotypes. Difference haplotype frequencies were calculated with Pearson's chi-squared test between case and control groups. $P \leq 0.05$ was considered statistically significant in all cases, and the error level was taken as 0.05.

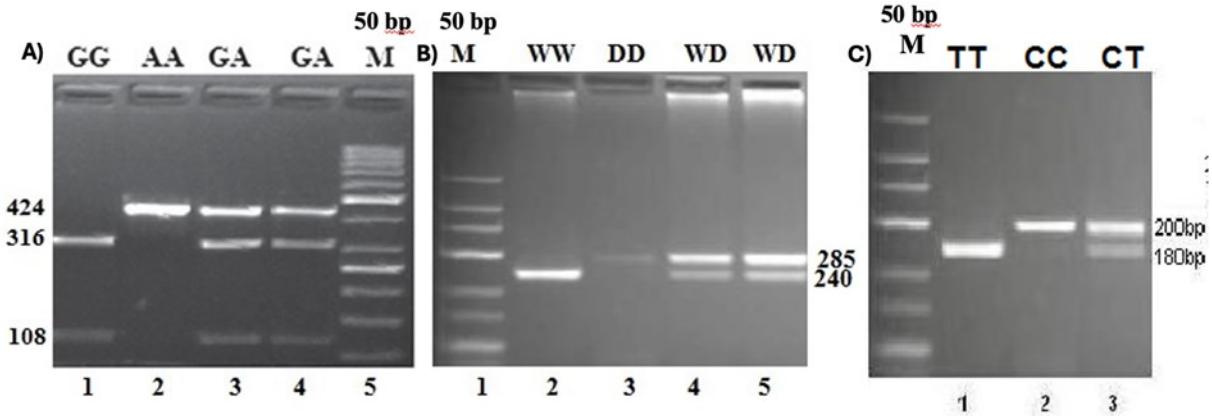

Figure 1: PCR-RFLP and sequence analysis of NF-κB1 W/D and NF-κB1A 3'UTR A→G and -826C/T polymorphisms. **A)** NF-κB1A 3'UTR A → G polymorphism, AA genotype: 424 bp number 2, GA genotype: 424, 316 and 108 bp; numbers 3 and 4, GG genotype: 316 and 108 bp; number 1. Marker: 50 bp; **B)** NF-κB1 -94W/D polymorphism, WW (ins/ins) genotype: 240 and 45 bp; number 2, WD (ins/del) genotype 285, 240 and 45 bp; numbers 4 and 5, DD (del/del) genotype 285 bp; number 3). M: 50 bp; **C)** NF-κB1A -826C/T polymorphism genotypes formed after cutting with Bfa I enzyme. Sample number 1 is TT genotype (20 bp + 180 bp). Sample number 2 is the CC genotype (200bp). Sample number 3 CT genotype (20bp+180bp+200bp). Marker: 50 bp.

RESULTS

In this study, we investigated the effect of 3 NF-κB gene polymorphism, which is a strong player in the immune response to COVID-19 disease. We compared allele and genotype frequencies of the study population regarding case-control, sex and severity (Table 1, Table 2, and Table 3, respectively). In this study, allele and genotype frequencies of the study group were compared. For the NF-κB1 -94 ins/del polymorphism, between case and control groups, we showed a statistically significant difference in the WD and DD genotype distribution ($p<0.001$, $p=0.038$, respectively). For the NF-κB1A 3'UTR A/G polymorphism, the G allele frequency had a 2.5-fold higher risk than the A allele ($p<0.001$; OR=2.52). For NF-κB1A -826 C/T polymorphism, the T allele frequency has a statistically significant difference ($p<0.001$) (Table 1). Haplotype analyses were also examined for all possible haplotypes. The data for all comparisons are also summarised in Table 1.

Table 1. Risk estimates and frequencies of haplotypes, alleles and genotypes for NF-κB (-94 ins/del, 3'UTR A/G, -826 C/T) polymorphisms in COVID-19 patients and healthy controls.

NF-κB1/B1A polymorphism		Case, n (%)	Control, n (%)	p-value	OR (95%CI)
-94ins/del	W allele	167 (55.96)	266 (66.17)	**0.005**	1.56 (1.14-
(rs28362491)	D allele	133 (44.04)	136 (33.83)		2.12)
	WW	35(23.33)	85 (42.29)		
Genotype	WD	97(64.67)	96 (47.76)	**0.001**	2.45(1.51-3.98)
	DD	18(12.00	20 (9.95)	**0.038**	2.19(1.03-4.62)
3'UTR A/G	A allele	111(37.00)	204(50.75)		
(rs696)	G allele	189(63.00)	138(49.25)	**0.001**	2.52(1.83-3.46)
	AA	21(14.00)	90(44.78)		
Genotype	AG	69(46.00)	84(41.79)	**0.001**	3.52(1.98-6.23)
	GG	60(40.00)	27(13.43)	**0.001**	9.52(4.93-18.37)
-826 C/T	C allele	154(51.33)	321(79.85)		
(rs2233406)	T allele	146(48.67)	81(20.15)	**0.001**	3.76(2.69-5.24)

Table 1. Continue.

Genotype	CC	42(28.00)	125(62.19)		
	CT	70(46.67)	71(35.32)	**0.001**	2.94(1.81-4.75)
	TT	38(25.33)	5 (2.49)	**0.001**	22.62(8.36-61.22)
Haplotypes	W A C	15(0.050)	24(0.133)		
	W A T	20(0.065)	8(0.045)	0.080	4.00(1.41-11.35)
	W G C	108(0.359)	57(0.314)	**0.002**	3.03(1.47-62.3)
	W G T	14(0.048)	20(0.111)	0.813	1.12(0.44-2.87)
	D A C	31(0.102)	16(0.087)	**0.011**	3.10(1.29-7.50)
	D A T	0(0.000)	13(0.080)	**0.011**	0.64(0.51-0.82)
	D G C	62(0.206)	34(0.187)	**0.005**	2.92(1.35-6.29)
	D G T	50(0.169)	8(0.043)	**0.001**	10.00(3.73-26.82)

Our study group consisted of 84 women and 132 men with COVID-19. We calculated genotype and allele frequencies between these groups and analyzed the statistical difference between them. However, we did not find a statistical difference due to gender for allele and genotype frequencies (Table 2)

One of the aims of this study was to compare the allele and genotype frequencies of these three SNPs in terms of COVID-19 severity. However, no statistically significant difference was observed between patients having severe COVID-19 in intensive care and outpatients (Table 3).

Table 2. Association between genotype and sex of the COVID-19 patients.

NF-κB1/B1A polymorphism		Female, n (%) (84)	Male, n (%) (132)	p-value	OR (95%CI)
-94ins/del	W allele	89(52.98)	78(59.10)		
(rs28362491)	D allele	79(47.02)	54(40.90)	0.290	1.28(0.81-2.03)
	WW	16(19.04)	19(28.79)		
Genotype	WD	57(67.86)	40(60.61)	0.183	1.70(0.78-3.68)
	DD	11(13.10)	7(10.60)	0.288	1.87(0.58-5.94)
3'UTR A/G	A allele	62(36.99	48(36.36)		0.98(0.80-1.59)
(rs696)	G allele	106(63.01)	84(63.64)	0.923	
	AA	10(11.90)	10(15.15)		
Genotype	AG	42(50.00)	28(42.42)	0.425	1.50(0.55-4.07)
	GG	32(38.10)	28(42.43)	0.796	1.14(0.41-3.15)
-826 C/T	C allele	83(50.00)	69(52.27)		
(rs2233406)	T allele	85(50.00)	63(47.73)	0.622	1.12(0.71-1.77)
Genotype	CC	25(29.77)	16(24.24)		
	CT	33(39.28)	37(56.06)	0.159	0.57(0.26-1.25)
	TT	26(30.95)	13(19.70)	0.597	1.28(0.50-3.19)

Table 3. Association between severe and mild COVID-19 patients.

NF-κB1/B1A polymorphism		Severe (%)	Mild (%)	p-value	OR (95%CI)
-94ins/del	WW genotype	5(33.33)	32(23.70)		
(rs28362491)	WD genotype	8(53.33)	88(63.18)	0.367	0.58(1.77-1.96)
	DD genotype	2(13.34)	15(11.11)	0.859	0.85(0.14-4.91)
3'UTR A/G	AA genotype	2(13.33)	19(14.07)		
(rs696)	AG genotype	5(33.33)	64(47.41)	0.733	0.74(0.13-4.31)
	GG genotype	8(53.34)	52(38.52)	0.648	1.46(0.28-7.50)
-826 C/T	CC genotype	4(26.67)	33(24.44)		
(rs2233406)	CT genotype	7(46.67)	76(56.30)	0.677	0.76(0.21-2.77)
	TT genotype	4(26.66)	26(19.26)	0.750	1.27(0.29-5.65)

DISCUSSION AND CONCLUSION

The COVID-19 pandemic is a viral infection that has become a public health problem in a short time and consists of various clinical stages. Patients may develop serious complications if a balanced immune response to viral infection is not established in the early stages. Acute Respiratory Distress Syndrome caused by the cytokine storm in COVID-19 has been shown to be an important cause of death. Therefore, suppressing the cytokine storm is critical to reduce mortality in COVID-19 patients. Activated transcription factors, including NF-κB, are activated to stimulate cytokine genes. Thus, released cytokines limit viral spread through paracrine effects as well as IFN-mediated gene stimulation.[15] Since NF-κB triggers the production of acute inflammatory mediators in various cells, it has been used in many in vivo and in vitro studies to elucidate the pathogenesis of respiratory viral infections and lung-related diseases.[16] NF-κB is an important regulator of differentiating

and activating T cells and other innate immune cells. Therefore, dysregulation of NF-κB can lead to an uncontrolled and pathogenic inflammatory response. Interestingly, upregulation of NF-κB was observed to be involved in developing *SARS-CoV-2* infection.[17] *SARS-CoV-2* can trigger an uncontrolled inflammatory response. Since NF-κB is involved in the inflammatory process, it is especially important to find compounds that will prevent the activation of this pathway. Therefore, this study aimed to discuss the role of a variant of NF-κB genes in the pathogenesis and treatment of COVID-19.

In this study, we examined the association between the severity of COVID-19 disease and NF-κBI -94 ins/del, NF-κBIA 3'UTR A→G, NF-κBIA -826 C/T polymorphisms. With this study, allele and genotype frequencies of NF-κBI -94 ins/del, NF-κBIA 3'UTR A→G, and NF-κBIA -826 C/T were determined for the first time in a Turkish population. In addition, it was determined whether there was a statistical difference between male and female individuals in terms of genotype and allele distributions. In addition, haplotype distributions and whether there is a statistical difference for these SNPs in COVID-19 disease were also determined.

The distribution of the mutant D allele in the NF-κBI -94 ins/del polymorphism was studied in different populations, and it was found that the frequency of this allele varied from 32% to 60%, and this frequency was 33.83% in our study.[8,18] Considering whether there was a statistically significant difference, our findings showed that the allele frequency of the D allele was significantly higher in the case group (44.04%), and the D allele may be a risk allele for COVID-19 (Table 1). There was also no significant difference in the disease severity between males and females (Table 2 and Table 3). Looking at some studies conducted in recent years, it was found that the D allele frequency was 38.5% in the Polish population,[19] 48.6% in the Indian population,[20] and 60% in the Western Chinese population.[21] We found a significant difference in WD and DD genotypes between COVID-19 patients and controls ($p<0.001$, $p=0.038$, respectively) (Table 1). We also found that individuals with WD and DD genotypes had approximately 2.5 higher risk for COVID-19 than individuals with WW genotype (odds ratio=2.45, odds ratio=2.19, respectively) (Table 1). A study found that the DD genotype significantly increased the risk of HCV infection compared to the WW genotype in rs28362491 polymorphism.[22] In another study conducted in line with our study, the DD genotype increased the risk in individuals with persistent HCV infection.[23] In a study conducted by Arslan et al.,[13] a comparison of WW genotypes with both WD and DD genotypes revealed that the difference between Crimean Congo Hemorrhagic Fever patients and controls was statistically significant.[13]

Regarding the NF-κB1A 3'UTR A/G polymorphism, mutant G allele distributions have been studied in different populations to date, and the frequency of this allele has been found between 38% and 52%. The mutant G allele frequency was found to be 38.7% in Alzahra,[24] 51.9% in Morocco, and [23] and 45% in Northern Spain.[25] Regarding NF-κB1A 3'UTR A/G polymorphism, both alleles and genotypes were found to be significant in COVID-19 patients compared to reference alleles and genotypes. The mutant G allele was statistically significant between patients and controls, and individuals carrying the G allele were found to be 2.5 times more at risk than individuals carrying the A allele ($p<0.001$, OR=2.52) (Table 1). We found a statistically significant difference in AG and GG genotypes between COVID-19 patients and controls ($p<0.001$; $p<0.001$) (Table 1). We also found that individuals with AG and GG genotypes had a 3.5-fold and 9.5-fold higher risk compared to individuals with AA genotype (OR=3.52; OR=9.52; respectively) (Table 1). Recently, a study conducted by Camblor and colleagues found that the NF-κBIA rs696 GG genotype was statistically significantly increased in the patient group compared to healthy population controls.[24] Remarkably, bronchoalveolar lavage samples from patients with critical COVID-19 compared with non-COVID-19 pneumonia and normal lung identified NF-κBIA as an upregulated gene in this disease.[26] The NF-κBIA gene encodes IκBa, a key inhibitor of NF-κB signalling that acts by blocking the translocation of RelA/p50 active dimers to the nucleus.[7] The G allele has been associated with decreased NF-κBIA mRNA stability and lower inhibitory activity in vitro.[27,28] Less inhibition of rs696 G allele, NF-κB may promote proinflammatory signalling of the pathway, thereby increasing the risk of COVID-19 complications and intensive care unit admission. Another viral disease study observed a positive correlation between NF-κBIA polymorphisms and disease progression, impaired liver function, and elevated serum levels of the cytokines TNF -α and IL-6 in patients with chronic HBV.[29] Based on our findings, together with those of previous studies, mutations in some NF-κBIA polymorphisms may lead to decreased NF-κBIA activity and subsequent overexpression of NF-κB, followed by activation of NF-κB, which further intensifies liver injury by inducing secretion of multiple cytokines.

In studies, the mutant T allele in NF-κB1A -826C/T polymorphism was found at different frequencies. This allele distribution was 6.2% in Romania.[29] In the Turkish population, this allele was 20.15%. Statistically significant differences were found in NF-κB1A -826C/T polymorphism in terms of both allele and genotype distribution. In the patient group, the T

allele was found to be statistically significant compared to the control group, and individuals with the T allele were found to be approximately four times more risky than individuals with the C allele (Table 1). Regarding genotype distributions, individuals with CT and TT genotypes were statistically significant compared to individuals with CC genotypes. Individuals with CT and TT genotypes were approximately 3-fold and 23-fold at risk for COVID-19 disease compared to individuals with CC genotype (Table 1, respectively). Lin et al.[8] proposed that mutations in some NF-κBIA polymorphisms may lead to reduced NF-κBIA activity and subsequent overexpression of NF-κB. Subsequent activation of NF-κB would further intensify liver damage by inducing the secretion of multiple cytokines. A study showed that rs2233406 increased the risk of developing autoimmune or inflammatory diseases 2.11-fold.[30] In our study, in line with these data, it was shown that individuals with TT genotype in rs2233406 polymorphism had a 23-fold higher risk of developing COVID-19 disease compared to individuals with CC genotype, and this risk was statistically significant (Table 1). Our haplotype analysis also showed that WGC, DAC, DAT, DGC, and DGT haplotypes were important in the risk of developing COVID-19 (Table 1). Individuals with the DGT haplotype have a 10-fold higher risk of developing COVID-19 (Table 1; $p<0.001$, OR=10.00).

In our study, we also compared COVID-19 on the basis of gender and severity of the disease, but we did not find a statistical relationship between the disease and gender. Likewise, we did not find any relationship in the comparison made according to severity.

In conclusion, allele and genotype frequencies of NF-κB1-94ins/del, NF-κB1A 3'UTR A/G, and NF-κB1A -826 C/T polymorphisms were higher in COVID-19 patients compared to healthy controls. NF-κB1-94ins/del, NF-κB1A 3'UTR A/G and NF-κB1A -826 C/T mutant genotypes may increase susceptibility to COVID-19 disease. However, more comprehensive studies are needed for these polymorphisms, especially in terms of protein levels or gene expression levels.

Ethics Committee Approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Sivas Cumhuriyet University (Decision No: 2021-02/07).

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

Author Contributions: Concept NÖŞ, BB; Supervision NÖŞ, SA, MB; Materials SB, MB; Data Collection and/or Processing – NÖŞ, BB, SB; Analysis and/or Interpretation –NÖŞ, BB, SB; Writing –NÖŞ, BB, SB, SA, MB.

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