DOI: 10.29345/futvis.64 Gelecek Vizyonlar Dergisi (fvj: Future Visions Journal) 3(1): 2019, 35-40

Relationship Between Apolipoprotein E Gene Polymorphism and Lipid Parameters in Patients with Behçet's Disease

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Abstract

Behcet's Disease (BD) is a rare, autoimmune, chronic and inflammatory disorder whose cause is not fully understood. This study was aimed to determine apolipoprotein E gene (*APOE*) polymorphism, and its relationship with serum lipid levels in BD. The study group consisted of 30 BD patients (18 male, 12 female) and 51 healthy volunteers (29 male, 22 female). Levels of serum lipids were determined by enzymatic, and apolipoproteins by the immunonephelometric methods. Genetic analysis of the *APOE*polymorphism was carrried out using the polymerase chain reaction (PCR)-based assay. Although the levels of total and low density lipoprotein cholesterol (TC and LDL-C respetively), apolipoprotein B (APOB) and erythrocyte sedimentation rate (ESR) had significantly ($p<0.05$) higher in patients than healthy volunteers, the levels of APOA-I, APOE, triglyceride (TG), high density lipoprotein cholesterol (HDL-C), polymorphonuclear leukocytes (PMNL) and frequencies of APOE alleles were not significantly different in both healthy and in patient groups.In conclusion, *APOE* gene polymorphism could not show a distinctive feature in BD patients.

Keywords: Behçet's disease, Apolipoprotein E polymorphism, lipid

Behçet Hastalarında Apolipoprotein E Gen Polimorfizmi ile Lipid Parametreleri Arasındaki İlişki

Özet

Behçet Hastalığı (BH), nedeni tam olarak aydınlatılamayan nadir görülen, kronik, otoimmün ve inflamatuar bir hastalıktır. Bu çalışma, BH'da apolipoprotein E gen (*APOE*) polimorfizmi ve serum lipid seviyeleri ile ilişkini belirlemeyi amaçlanmıştır. Çalışma grubunu 30 hasta (18 erkek, 12 kadın) ve 51 sağlıklı gönüllü (29 erkek, 22 kadın) oluşturdu. Serum lipid seviyeleri enzimatik ve apolipoproteinler immünonefelometrik yöntemlerle belirlendi. *APOE* polimorfizminin genetik analizi, polimeraz zincir reaksiyonu (PCR)'a dayalı analiz ile gerçekleştirildi. Toplam ve düşük dansiteli lipoprotein kolesterol (sırasıyla TC ve LDL-C), apolipoprotein B (APOB) düzeyleri ve eritrosit sedimentasyon hızı (ESR), hastalarda sağlıklı gönüllülerden anlamlı ($p <0.05$) olarak daha yüksek bulunmasına rağmen, apolipoprotein A-I(APOA-I), APOE, trigliserit (TG), yüksek dansiteli lipoprotein kolesterol (HDL-C), polimorfonükleer lökositler (PMNL) ve Apo E allellerinin frekansları, hem sağlıklı hem de hasta gruplarında anlamlı olarak farklı değildi. Sonuç olarak *APOE* polimorfizmi Behçet hastalığında herhangi bir ayırt edici özellik göstermemiştir.

Keywords: Behçet hastalığı, Apolipoprotein E polimorfizmi, lipid

1 Introduction

Behcet's disease (BD) described firstly by Hulusi Behçet is known as a chronic and multisystem inflammatory disorder seen rarely (Yazıcı, et al.,2010). In recent years, it is described as a chronic immune-mediated, inflammatory disorder with an etiology of oral and genital ulcerations, skin and ocular lesions, and other manifestations such as neurological and gastrointestinal tract involvement (Greco et al,.2018). The pathogenesis of BD remains poorly understood, but genetic, environmental, and autoimmune system abnormalities have been reported as the major determinants (Kul et al.,2017). The triggering of infectious factors also contributes to outbreaks of BD in genetically predisposed patients. Stimulation of chemotaxis, phagocytosis, oxidative stress and lysosomal enzymes also causes an increase in PMNL cells in BD (Pineton de Chambrun et al.,2012).

APOE is a multifunctional protein involved in lipoprotein transport, immunoregulation, nerve regeneration, tissue repair, and cognitive functioning (Razali et al,.2013). It has a function in cellular cholesterol uptake, the proliferations of myeloid cells and the activation of monocytes and infiltration of them into the vascular walls. Thereby it suppresses atherosclerosis. APOE is also known to affect the macrophages the polarity and inflammatory phenotypes, as well as regulating innate immunity response to bacterial infection (Raffai, 2012). *APOE* gene of human is located on 19th chromosome and exists as three polymorphic alleles: ε4, ε3 and ε2 (coding for three isoforms: E4, E3, and E2; producing three homozygous E4/E4, E3/E3, E2/E2 and three heterozygous E3/E4, E2/E4, E2/E3) (Tanguturi et al,.2013). Only amino acid substitution among the three APOE isoforms alter the protein's structure and affect its lipid and receptor binding properties (Zhong et al.,2016). In contrast to ε4, ε2 has been shown to be associated with higher serum levels of APOE, and lower serum levels of LDL-C (Karahan et al.,2015). However, some researchers have reported no relationship between the ε4 allele and lipid levels. These different findings may due to *APOE* gene polymorphism varying within and between ethnic groups. For example, while Europeans have a high frequency of ε4, this is much lower in Asians (Larifla et al.,2017).

The protective effects of HDL and its major protein APOA-I are largely attributed to their ability to mediate cholesterol efflux from peripheral cells. Furthermore, their antioxidant, anti-inflammatory and antithrombotic properties contribute to exhibit antiatherogenic effects (Montecucco et al.,2015). Apolipoprotein B100 (Apo B100) is a key protein component of LDL and a ligand for LDL receptors in uptaking of LDL from peripheral cells and the liver. Therefore it has very important functions in cholesterol homeostasis (Biswas et al.,2013). Given that allele frequencies and polymorphisms differ among ethnic groups, the present study aimed to assess *APOE* polymorphism and its association with the levels of serum lipids and apolipoproteins (APOA, B and E) in patients with BD in the Black Sea Region of Turkey.

2 Material and Methods

2.1 Study Group

The study groups included 30 BD patients (18 men/12 women; at age 23-51 years) and 51 age- and sex-matched healthy volunteers (29 men/22 women; at age 20-45 years). Clinical diagnoses of BD, according to the criteria formed by the International Study Group for BD, were identified in Dermatology Department, Faculty of Medicine, Karadeniz Technical University. Patients with oral and genital aphthous ulceration, ocular involvement, erythema nodosum, vascular involvement and arthritis were included. However, patients receiving any corticosteroid or hormone replacement therapy, with a history of alcohol consumption of smoking, or with diabetes mellitus, renal, coronary or liver failure, or additional autoimmune disorders, were excluded from the study. Bloods were collected from patients who did not receive any treatments (for at least one month). The control group consisted of healthy volunteers who did not have any other current disease and alcohol consumption or smoking. Pregnant women were also excluded.

The current study was managed in accordance with, the principles of the Declaration of Helsinki (Wechsler& Davatchi, 1990). Samples were collected in between 1997 and 2000 in accordance with the relevant guidelines and ethical protocols, and informed consents from the patients and controls were obtained before their enrolments in the study.

2.2 Determinations of the Levels of Lipid Parameters and PMNL

After collecting of 12-h fasting whole blood samples of all study subjects, serum samples were obtained by centrifugation at 2000 g for 10 min. Serum glucose, TC and TG levels were measured using enzymatic methods on a Hitachi 917 autoanalyzer (Roche Diagnostic, Mannheim, Germany) by using its original reagents. HDL-C levels were calculated using the dextran sulfate-Mg^{2+} precipitation method. LDL-C levels were determined using the formula described by Friedewald (Friedewald et al.,1972). The levels of APOE, APOA-I and B were assayed the immunonephelometrically by using a BN II autoanalyzer (Dade Behring, Marburg, Germany). In the ethylenediamine tetraacetic acid (EDTA) anticoagulated whole blood samples, ESR and PMNL were determined by the classic Wintergreen method and by an automated blood cell counter (STKS, Coulter) respectively.

2.3 Determination of the APOE Polymorphisms

Genomic DNA from leukocytes in whole blood samples (collected into EDTA tubes) was extracted by using a standard salting-out method (Miller et al., 1988). The yields and purities of the DNA samples were assessed from assays of OD_{260}/OD_{280} performed with a spectrophotometer (Shımadzu UV-1601, USA). Next, DNA was visualized on 0.8% agarose gel (A-6877, Sigma) by ethidium bromide staining (E1510, Sigma). After that, amplification of this DNA was done by PCR with the primers F5'-CCAAGGAGCTGCAGGCGGCGCA and R5'-GCCCCGGCCTGGTACACTGCCA (Genemed Biotechnologies, USA) to yield a 218-bp double-stranded DNA fragment. In the PCR technique, 100-200 ng/μL of DNA was added to 40 μL of reaction mixture: 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 0.36 μM of each primer, 400 μM dNTP, 50% dimethyl sulfoxide (Sigma), and 1 U of Taq polymerase (Promega, M1861). The PCR reactions were then subjected to 40 cycles in a thermal cycler apparatus (Techne, UK) with 60 s of denaturing at 94°C, 60 s of annealing at 55°C, and 90 s of extension at 70°C. Amplification control was performed using the molecular weight standard pUC 19 DNA /MspI (hpa II) (MBI, AM0221) on 2% agarose gel. Amplified DNA (10 μL) was digested simultaneously with 5 U of *AflII* (Roche Applied Science, Germany) and 5 U of *HaeII* (Boehringer-Mannheim, Germany) for 4 hours at 37°C (Zivelin et al,.1997). Fragments of *APOE* alleles observed after cutting of the restriction enzymes in 7.5% polyacrylamide gel are illustrated in Figure1. The fragments produced for each alleles are as follows:

ε2 allele: 112Cys+ 158Cys=168 bp+50 bp

ε3 allele:112Cys+158Cys=145 bp+50 bp+23 bp

ε4 allele: 112Cys + 158Cys=195 bp + 23 bp.

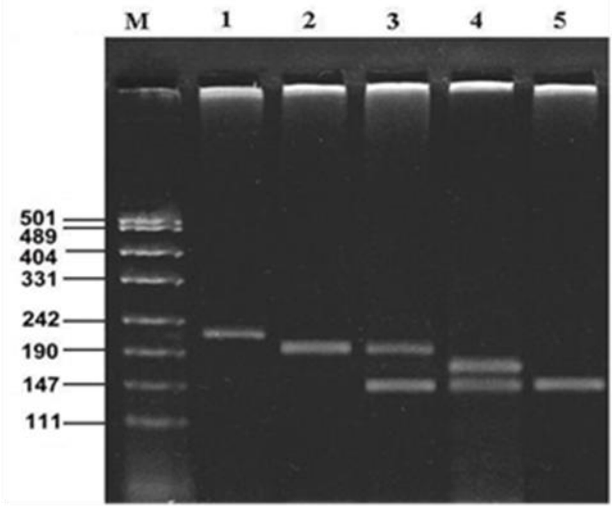

Figure 1. Fragments of APOE alleles after cutting of the restriction enzymes.
M: Molecular weight standard; 1: 218 (bp) product; 2: ε4/ε4 genotype; 3: ε3/ε4 genotype; 4: ε2/ε3 genotype; 5: ε3/ε3 genotype.

2.4 Statistical Analysis

After the testing of the distributions of variables by using the Kolmogorov-Smirnov test (KS-test), student's t-test was applied to compare the means of the study groups. Data were given as mean ± standard deviation (SD). Relations between variables were determined by using Spearman's rank correlation. In both groups, genotype frequencies were compared by the Chi-square test and allele frequencies by The Kruskal-Wallis test. Statistics was accepted significant when value of $p<0.05$.

3 Results and Discussion

Insignificant differences of age, sex and body mass index [BMI] of the patient and control groups were obtained (Table 1). The levels of TC, LDL-C, APOB and ESR were higher than the control subjects significantly ($p<0.05$). Insignificant difference was obtained in APOA-I, APOE, TG, HDL-C and PMNL levels (p>0.05) (Table 1). In the present study, PMNL (not significant) and ESR (significant) levels were higher in patients group (Table 1). That increased levels of ESR may be a sensitive marker of inflammation associated with the pathogenesis of BD. Because the inflammatory mediators are released into cytoplasm when phagocytic cells (neutrophils and macrophages) are stimulated (Abdulghafur & Rasool, 2017).

Table 1. Demographic characteristics and the levels of biochemical parameters in the study groups

	BD(*n=30*)	Controls(*n=51*)	*p*
Sex (M/F)	38±8.6	35±7.7	-
Age (years)	38±8.6	35±7.7	-
BMI (kg/m^2)	25±3.8	24±3.3	-
APOA-I (mg/dL)	120±22	129±18.7	0.600
APOB (mg/dL)	91±22	78±21.0	0.009
APOE (mg/dL)	3.7±0.7	3.9±0.9	0.312
TC (mg/dL)	200±41	178±28	0.005
TG (mg/dL)	133±51	114±45	0.078
HDL-C (mg/dL)	45±8.2	47±8.5	0.302
LDL-C (mg/dL)	128±38	111±32	0.030
PMNL (10^3/μL)	8.1±4.8	6.0±2.0	0.067
ESR (mm/h)	18.6±17.4	7.5±5.3	0.000

Significant correlations were observed between APOB and TC, TG, and LDL-C levels; between APOA-I and HDL-C; and between TC with LDL-C in both study groups. But there was statistically insignificant correlation of APOE levels with TC, LDL-C, TG and APOB in patients group (Table 2).

A study investigating the levels of lipids in BD demonstrated the increased levels of β-lipoproteins (Ohguchi et al.,1982). Mitamura et al.(1988) researched lipid parameters in BD too and found the decreased APOA-I and HDL-C levels but insignificant difference in TC and TG levels. Another study with BD showed the lower HDL-C but higher TC and TG levels (Esmat et al.,2006). Tursen et al. (2004) reported an increased level of HDL-C, but determined no significant difference in levels of TG, TC, VLDL-C or LDL-C in BD. They suggested that normal or increased HDL-C levels are both possible because coronary heart disease is uncommon in patients with BD. Örem et al. (2002) reported significantly increased levels of LDL-C, TC, APOB, and decreased levels of HDL-C, APOA-I, but observed no significant difference in TG levels in patients with BD. Finally they suggested that BD is associated with chronic active inflammation, and affects lipid metabolism during inflammation. Increased levels of TG and TC levels but decreased levels of HDL-C and phospholipids are the characteristic disturbances seen during the acute phase response (Messedi et al.,2011). However, the decreased levels of some lipids and lipoproteins may be observed by increasing inflammatory activity, due to the increased elimination through the reticuloendothelial system (Musabak et al.,2005). The inconsistency between these studies may be due to variation in the clinical activities of the randomly selected patients.

Table 1. Correlations (r) of apolipoproteins with serum lipids in the study groups

	APOE	APOB	APOA-I
Patients with BD			
TC	0.54 (0.002)	0.68 (0.001)	-
TG	0.47 (0.008)	0.47 (0.009)	-
LDL-C	0.48 (0.007)	0.62 (0.001)	-
HDL-C	-	-	0.65 (0.001)
APOB	0.53 (0.002)	-	-
Control subjects			
TC	0.44 (0.001)	0.48 (0.001)	-
TG	-	0.37 (0.007)	-0.31 (0.026)
LDL-C	0.31 (0.025)	0.53 (0.001)	-
APOB	-	-	-0.30(0.031)

Values were given as *r* (*p*): relation (probability)

The differences of *APOE* allele frequencies and genotypes and were not significant in our study (Table 3). Genotype ε4/ε4 was seen in only one patient, while ε2/ε2 and ε2/ε4 were not seen in any subjects. The ε2, ε3 and ε4 alleles frequencies in all samples (BD and control) were 10%, 73% and 17%, respectively. Most subjects were ε3/ε3 homozygotes (n=44; 54%), while 22 subjects (27%) were ε4 carriers who have at least one ε4 allele, and 15 subjects (18%) were ε2 carriers who have at least one ε2 allele. No significant difference was determined in *APOE* genotype distribution between study groups (Table 3).

Table 2. *APO E* genotypes and allele frequencies in BD and control subjects

	Patients n (%)	Controls n (%)
APOEgenotypes frequency		
ε3/ε3	15 (50.0)	29 (56.9)
ε3/ε4	8 (26.7)	13 (25.5)
ε2/ε3	6 (20.0)	9 (17.6)
E4/4	1 (3.3)	-
APOE allele frequency		
ε2	6 (10.0)	9 (8.8)
ε3	44 (73.3)	80 (78.4)
ε4	2(16.7)	13(12,8)

ε2/ε2 and ε2/ε4 genotypes were not detected in our study groups. No significant difference in frequencies was also seen between the groups (x^2=1.930, df=3). Lipid, lipoprotein and apolipoprotein levels classified by genotypes in the patient and the control groups were shown in Table 4. Insignificant difference was detected among genotypes in either group.

Table 4. The levels of serum lipids and lipoproteins according to *APOE* genotypes in BD and control subjects

	Patients with BD					Controls			
	ε3/ε3 (*n=15*)	ε3/ε4 (*n=8*)	ε2/ε3 (*n=6*)	ε4/ε4 (*n=1*)	*p*	ε3/ε3 (*n=29*)	ε3/ε4 (*n=13*)	ε2/ε3 (*n=9*)	*p*
APOE	3.6±0.7	3.9±0.8	3.7±0.5	2.5	0.46	4.0±0.9	3.8±0.9	3.7±0.7	0.39
APOA-I	121±28	124±16	118±9	91	0.41	128±20	130±15	130±21	0.84
APOB	92±20	93±15	90±37	75	0.75	80±22	78±22	69±15	0.35
TC	209±46	191±31	192±43	185	0.66	182±32	173±25	170±22	0.55
TG	137±58	134±26	124±38	141	0.94	119±50	119±42	94±25	0.31
HDL-C	44±8.0	45±10	46±7.8	39	0.89	46±8.0	47±10	47±9.0	0.98
LDL-C	137±43	119±26	120±43	118	0.65	115±35	106±33	104.3±24	0.72

One of the functions of APOE is to participate in lipoprotein transport and in mediating cellular cholesterol efflux (Biswas et al,.2013). Our findings of correlation with APOE were therefore not unexpected. We found only one previous study that investigates *APOE* polymorphism in BD (Tursen et al.,2004). Similar to our study, they found no difference in *APOE* polymorphism (in terms of genotypes and alleles). They concluded on the basis of their results that despite its antioxidant and antimicrobial activities, APOE plays no role in the pathogenesis of BD. However, several studies have investigated *APOE* polymorphism in other inflammatory diseases. *APOE3* having ε3 allele imparts the anti-inflammatory and antioxidative properties while *APOE4* having, the ε4 allele imparts pro-inflammatory properties and increases the risk for both cardiovascular and neurodegenerative diseases (Kuhel et al,.2013). Hultman et al. (2013) reported that the *APO*ε4/ε4 genotype increased thrombosis and/or impaired fibrinolysis in patients with Alzheimer's disease. Tanguturi et al. (2013) found a 1.5-fold greater generality of ε3/ε4 genotypes in subjects with myocardial infarction (MI) compared to controls, but that the generality of ε2/ε3 genotypes was higher in the controls compared to the patient groupThey also reported that the ε4 allele was significantly related with MI. Toms et al. (2012) determined that the frequency of *APOE* polymorphisms had not significant in patients with rheumatoid arthritis, and concluded that *APOE* genotypes are strongly linked to inflammation and lipid levels in rheumatoid arthritis. Therefore the detection of *APOE* genotypes in a familial BD may be more useful informative in this topic.

The most important limitation of the current study was the limited number of patients. Because of the low prevalence of BD in population, insufficient patients were found. To identify whether ε4/ε4 genotypes seen in one patient with BD but not seen in control subjects is fortuitous, large number of patients were needed. Another limitation of the study is the variety in the clinical activity stages of the patients.

4 Conclusion

In the current study examining the *APOE* gene polymorphisms in BD, the levels of lipids and lipoproteins according to *APOE* alleles were not different significantly although the levels of the lipid parameters were different from control subjects. It was concluded that although the levels of atherogenic lipids affected, the frequencies of *APOE* alleles and polymorphism were not significant in BD living in Karadeniz Region in Turkey. This study need to be supported with large number of subjects.

Acknowledgements:

This study supported by Scientific Research Projects Unit of Karadeniz Technical University (Text.project number 97.114.001.1). We would like to thank Prof. PhD, Gülseren Çimşit, (Department of Dermatology, Karadeniz Technical University, Medicine Faculty) due to the contribution to the collection of plasmaof the patients with BD.

Declaration of interest

The authors report no conflict of interest.

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