HTRA1 and CFH Gene Polymorphisms in Turkish Patients with Exudative Age Related Macular Degeneration

Eksudatif Yaşa Bağlı Makula Dejenerasyonu Olan Türk Hastalarda HTRA1 ve CFH Gen Polimorfizmleri

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ABSTRACT

Purpose: To investigate the genes HTRA serine peptidase1 (HTRA1) and complement factor H (CFH) in Turkish patients with exudative age-related macular degeneration (AMD).

Materials and Methods: This study included 39 exudative AMD patients and 250 healthy individuals with exome sequencing data as a control group. Patients with known environmental and systemic risk factors for AMD were excluded. Genomic DNA was isolated from peripheral blood and analyzed using next-generation sequencing. All coding exons of the HTRA1 gene and selected exons (3, 9 and 10) of the CFH gene were analyzed.

Results: Sequence analysis of the CFH gene identified two genetic variations in the study group. Our results identified these variations as polymorphisms rs1061170 (H402Y) and rs2274700 (A473A). Additionally, rs369149111 (A20V) polymorphism, in which a non-synonymous amino acid exchange in exon 1 of the HTRA1 gene was detected. This non-synonymous exchange was a single nucleotide polymorphism and did not lead to a pathological condition based on PolyPhen and SIFT analyses.

Conclusion: The study showed that heterozygous variations of the risk alleles of rs1061170 (H402Y) and rs2274700 (A473A) polymorphisms of the CFH gene were associated with AMD risk, compared to the homozygous variant of the normal allele in Turkish patients.

Keywords: Age-related macular degeneration, gene polymorphism.

ÖZ

Amaç: Eksudatif yaşa bağlı makula dejenerasyonu (YBMD) olan Türk hastalarda, HTRA1 (HTRA serine peptidase1) ve CFH (complement factor H) genlerinin incelenmesi.

Gereç ve Yöntem: Bu çalışmaya 39 eksudatif YBMD hastası ve kontrol grubu olarak ekzom dizileme datası olan 250 sağlıklı birey dahil edildi. Senil makula dejenerasyonu açısından bilinen çevresel ve sistemik risk faktörlerinin olduğu olgular çalışmaya alınmadı. Genomik DNA periferik kandan izole edildi ve yeni nesil dizileme tekniği ile analiz edildi. HTRA1'in tüm kodlayıcı eksonları ve CFH'nin seçilmiş eksonları (3, 9 ve 10) incelendi.

Bulgular: Çalışma grubunda CFH'nin sekans analizi ile 2 genetik varyasyon bulundu. Sonuçlarımıza göre bu varyasyonlar rs1061170 (H402Y) ve rs2274700 (A473A) polimorfizmleri idi. Buna ek olarak, HTRA1 geninin 1. eksonunda özdeş olmayan aminoasid değişimi olan rs369149111 (A20V) polimorfizmi saptandı. Bu özdeş olmayan değişiklik tek bir nükleotid polimorfizmi idi ve PolyPhen and SIFT analizlerinde bir patolojik duruma sebep olmuyordu.

Sonuç: Bu çalışmada Türk hastalarda, CFH geninin rs1061170 (H402Y) ve rs2274700 (A473A) polimorfizmlerinin risk alellerinin heterozigot varyasyonlarının, normal alellerin homozigot varyasyonlarına kıyasla YBMD riski ile anlamlı ilişkisi saptanmıştır.

Anahtar Sözcükler: Yaşa bağlı makula dejenerasyonu, gen polimorfizmi.

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in individuals older than 65 years of age. AMD is a multifactorial disease and the risk factors include age, ethnicity, smoking, hypertension, obesity, sunlight exposure and genetic properties.[1] Approximately 50% of the etiology can be attributed to the genetic variations in the complement factor H (*CFH)*, age-related maculopathy susceptibility protein 2 (*ARMS2*), and interleukin-8 (*CXCL8/IL-8*) genes, which encode CFH, ARMS2, and IL-8, respectively. Genetic variations in the complement pathway, such as *CFH*, angiogenesis pathway such as vascular endothelial growth factor (*VEGF*), high-density lipoprotein metabolic pathway such as cholesteryl ester transfer protein (*CETP*), as well as HTRA serine peptidase 1 (*HTRA1*) were reported to be associated with AMD.[2-5]

The *CFH* gene located on the chromosome 1q32 was the first identified gene to be associated with AMD.[6-8] It has been shown that the disappearance of the CFH protein affects complement inhibition, leading to excessive inflammation and tissue damage, which are responsible for the pathogenesis of AMD.[9]

The *HTRA1* gene located on chromosome 10q26 encodes a member of the serine protease family expressed in the retina and retinal pigment epithelial cells. Serine proteases inhibit the pathways mediated by the transforming growth factor-beta (TGF-β) family. It has been reported that changes in the TGF-β signal pathways alters the regulation of retinal angiogenesis.[10] Changes in the expression of *HTRA1* may lead to pathological events in the Bruch's membrane layer, which have been suggested as an etiological factor for the development of AMD.[11]

In this study, we aimed to investigate the *HTRA1* and *CFH* genes in Turkish patients with exudative (wet) AMD using next-generation sequencing (NGS).

MATERIALS AND METHODS

Patients

This study included 39 patients with exudative AMD. Patients were excluded from the study if they had a history of smoking, previous ocular trauma, excessive sunlight exposure, systemic cardiovascular diseases, and choroidal neovascular membrane not caused by AMD. The exudative AMD diagnosis was based on the detailed ophthalmological examination including biomicroscopy, dilated fundus examination, optical coherence tomography and fluorescein angiography. The study was approved by institutional review board and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Blood collection and DNA extraction

The blood samples were collected from antecubital vein at ophthalmology department of our institution. Along with the exudative AMD patients, the study included 250 healthy individuals with exome sequencing data as a control group. Blood samples were centrifuged at 5000 rpm for 3 minutes and the buffy coat was collected. Genomic DNA was isolated from buffy coat using Real Pure Spin Kit (Real TM, Durviz, Spain) according to the manufacturer's instructions. The DNA was eluted with 200 µL of elution buffer and stored at -20 °C until use. All exons of the *HTRA1* gene and selected exons (3, 9, and 10) of the *CFH* gene were analyzed using NGS.

Targeted NGS

Sequencing analysis of the *HTRA1* and *CFH* genes was performed using the MiSeq NGS platform (Illumina Inc., San Diego, CA, USA). The DNA samples were quantified with NanoDrop 1000 (NanoDrop, Wilmington, DE, USA) spectrophotometer and used at a concentration of 50 ng/µL. All exons of *HTRA1* gene and exons 3, 9, and 10 of the *CFH* gene as well as their flanking splice site junctions were amplified using PCR primers that were designed with PRIMER©-Primer Designer v.2.0 software (Scientific & Educational Software Programme). Agarose gel electrophoresis was used to validate the PCR results. The PCR products for each sample were mixed to obtain PCR pools, purified, and quantified. Purifications were performed using the NucleoFast® 96 PCR kit (Macherey-Nagel GmbH, Düren, Germany), and quantification of purified PCR products was done using NanoDrop 1000. Quantified PCR pools were then standardized to 0.2 ng/µL. The libraries were prepared with the NexteraXT kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. NGS was carried out on a MiSeq device (Illumina Inc., San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed using the SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). The strength of the associations between the three polymorphisms (rs1061170, rs2274700, and rs369149111) and the AMD risk was assessed by logistic regression analysis. Odds ratios with corresponding 95% confidence intervals were calculated. A p value <0.05 considered as statistically significant.

RESULTS

Patients

The patient group consisted of 15 female (38%) and 24 male (62%) individuals. The mean age of the patients was

75.7±7.8 years. Age and sex distribution of the control group was similar with that of the patient group (p>0.05 for both).

Genetic variations detected in the *HTRA1* gene

We detected one genetic variation in the patient group. Using PolyPhen and sorting intolerant from tolerant (SIFT) analyses, A20V (rs369149111) was identified as a single nucleotide polymorphism (SNP) with a non-synonymous substitution in exon 1. This polymorphism did not associate with a pathological condition. We determined five different genetic variations that were identified as SNPs in the *HTRA1* gene in the control group (Table 1).

Genetic variations detected in *CFH* gene

Sequence analysis of the *CFH* gene revealed two different genetic variations in the AMD patients. Our results identified these changes as polymorphisms rs1061170 (H402Y) and rs2274700 (A473A). We detected ten different SNPs on the *CFH* gene in the control group (Table 2).

We examined the association of AMD with the rs1061170 (H402Y) and rs2274700 (A473A) polymorphisms in the *CFH* gene with the multivariate analysis method (Table 3).

We determined that the heterozygous variations of the risk

Table 1. *Genetic variations in the HTRA1 gene in the patient and control groups.*

Group	Variation	Protein	dbSNP ID	MAF/1000 Genome Project	Allele Frequency	Number of Cases/Controls	
						Homozygous	Heterozygous
Patient (N=39)	c.59C>T	A20V	rs369149111	0.0507	0.0128		1
Control (N=250)	c.102C>T	A34=	rs1049331	0.2931	0.0500	1	23
	c.1274+14G>A	intronic	rs2272599		0.0820	12	17
	c.108G>T	G36=	rs2293870	0.2913	0.0100	1	3
	c.1275-36C>T	intronic	rs2293871		0.1340	6	55
	c.1274+8G>A	intronic	rs2672586		0.7160	179	

MAF: minor allele frequency

Table 2. *Genetic variations in the CFH gene in the patient and control groups.*

Group	Variation	Protein	dbSNP ID	MAF/1000 Genome Project	Allele Frequency	Number of Cases/Controls	
						Homozygous	Heterozygous
Patient (N=39)	c.1204C>T	H402Y	rs1061170	0.2666	0.3974	6	19
	c.1419G>A	A473=	rs2274700	0.479	0.2308		18
Control (N=250)	c.921A>C	A307=	rs1061147	0.2869	0.6220	106	99
	c.1204C>T	H402Y	rs1061170	0.2666	0.2580	45	39
	c.2808G>T	E936D	rs1065489	0.2033	0.1360	9	50
	c.1419G>A	A473=	rs2274700	0.479	0.1720	20	46
	c.3148A>T	N1050Y	rs35274867	0.0142	0.0120		6
	c.2634C>T	H878=	rs35292876	0.0064	0.0340	1	15
	c.2016A>G	Q672=	rs3753396	0.2029	0.1300	8	49
	c.3134-5T>A	intronic	rs513699		0.0520		26
	c.3138C>T	T1046=	rs61822181		0.0960		48
	c.184G>A	V62I	rs800292	0.4681	0.2120	14	78

MAF: minor allele frequency

Table 3. *Multivariate analysis* of the variations in the CFH gene among the patients.*

dbSNP ID	Variation	Allele Frequency		OR (95% CI)	p
		Control (N=250)	Case (N=39)		
rs1061170	c.1204C>T	0.258	0.398	5.777 (2.66 - 12.51)	<0.001
rs2274700	c.1419G>A	0.172	0.230	3.429 (1.690 - 6.957)	0.001

*Other variants were corrected in the logistic regression analysis.
OR: odds ratio, CI: confidence interval

alleles of rs1061170 (H402Y) and rs2274700 (A473A) polymorphisms were associated with the AMD risk compared with the homozygous variant of the normal allele.

DISCUSSION

Age-related macular degeneration is a multifactorial disease that can lead to significant vision impairment in elderly. The etiology includes both genetic and environmental factors. Concordance studies in monozygotic and dizygotic twins revealed that hereditability is one of the main genetic risk factors for AMD.[12] The hereditability was estimated to be at least 11% in presence of one affected first relative, and the AMD risk was shown to increase 2.4-fold compared to families without the disease.[13] Several studies performed between 2005 and 2007 reported that *ARMS2* and *CFH* were the major genetic factors responsible for AMD.[2,7,8,14,15]

Although the precise role of *CFH* on the pathogenesis of AMD has not been fully clarified, it has been charged to be a key immunity component in the formation of drusen.[16,17] Specific polymorphisms in complement genes (*CFH*, *C3*, *C2*, *CFI*, and *CFB*) were identified and determined to be associated with the risk of AMD.[18] It has been reported that polymorphisms in the *CFH* gene account for the high risk of exudative AMD and are also highly associated with non-exudative AMD.[8] In addition, an association of the *CFH* gene with geographic atrophy and choroidal neovascularization has also been reported previously.[6-8]

Studies in various populations have established the association of the *CFH* gene polymorphisms with AMD.[19,20] Liu et al. reported that four SNPs, including rs3753394, rs800292, rs1061170, and rs1329428 in *CFH* gene were significantly related with exudative AMD.[4] A meta-analysis by Holliday et al. revealed a strong association between the SNPs in the *CFH* and *ARMS2/HTRA1* loci with early AMD risk. They found that 6 SNPs (rs1329424, rs1061170, rs10737680, rs1410996, rs380390, and rs1329428) in *CFH* gene were associated with AMD.[21] Tian et al. evaluated the association between AMD and 15 SNPs in the *CFH* gene and found that 5 of them (rs551397, rs800292, rs10737680, rs2274700, and rs1410996) were found to be associated with increased risk of AMD when the risk alleles were homozygous. In addition, they showed that five other SNPs (rs1329424, rs1061170, rs10801555, rs10733086, and rs380390) were associated with AMD when the risk alleles were either homozygous or heterozygous.[22] Several studies have reported an association between the Y402H polymorphism (rs1061170) in exon 9 of the *CFH* gene and AMD. The risk allele frequency of rs1061170 was found to be 10.9% in the Chinese population whereas it was found to be 34.9% in the Caucasian population.[6,22] Our study revealed that the risk allele frequency in the Turkish population (39.8%) was similar to that in the Caucasian population. We did not find a significant association between the homozygosity of the risk allele and AMD, which could be attributed to the small sample size. The odds ratio (5.7, $p<0.001$) of the advanced AMD patients in the present study was consistent with that of previous studies in the literature.[21-23]

Familial AMD cohort studies have shown a strong association between the rs2274700 risk allele and AMD.[20,22,24] In the present study, none of the individuals in the patient group were homozygote for the rs2274700 risk allele. However, heterozygous individuals for this SNP were identified in the control group. We detected that the risk increased 3.4-fold in the individuals with a heterozygous rs2274700 risk allele compared with those who did not carry this allele. None of the subjects possessed homozygous rs2274700 risk alleles, which could also be due to the relatively small sample size.

The relationship between the *HTRA1* gene at 10q26 and AMD has been described in pedigree analysis and mapping and reported in several genome-wide association studies. In these gene loci, three gene clusters consisting of the *PLEKHA1*, *ARMS2*, and *HTRA1* were defined in a 140-kb region. It has been proposed that each of these genes was related to AMD, and polymorphisms in this region increase AMD risk.[5,13,14,25] Richardson et al. have also reported that the AMD was related to the polymorphisms at 10q26 and that the strongest association was with the rs3793917 polymorphism.[26] It has been shown that the rs10490924, rs3750848, del443ins54, rs3793917, rs11200638, and rs932275 polymorphisms located between the *ARMS2* and *HTRA1* genes also have strong associations with AMD.[15] Dewan et al. reported that rs11200638 in the promoter region of *HTRA1* was a major genetic risk factor for exudative AMD. They found that the individuals with the risk-associated genotype were estimated to have a 10-times greater probability of developing exudative AMD than individuals with the wild-type genotype.[5] Moreover, several other studies have demonstrated that rs11200638 is the most likely causal variant for AMD.[25,27] Chen et al. performed a meta-analysis of 14 case-control studies, and their subanalysis indicated that rs11200638 in *HTRA1* could increase the risk of AMD in all races.[28] We examined all of the exons in *HTRA1* and identified that the rs369149111 variant detected in the patient group was an SNP previously recorded in the National Center for Biotechnology Information (NCBI) and Human Genome Variation Society (HGVS) databases (We examined all of the exons in HTRA1 and identified that the rs369149111 variant detected in the patient group was an SNP previously recorded in the National Center for Biotechnology Information (NCBI) and Human Genome Variation Society (HGVS) databases (www.ncbi.nlm.nih.gov/projects/SNP/, www.hgvs.org/mutnomen/). However, we determined that it did not cause a pathologic change in the protein using PolyPhen and SIFT analyses.

There are some limitations in the present study, mainly the

small sample size and the limited gene analysis. We have included exudative AMD patients with Turkish ethnicity only. We have investigated only the *HTRA1* and *CFH* genes for possible exudative AMD association.

In conclusion, our study revealed that the heterozygous risk alleles for rs1061170 (H402Y) and rs2274700 (A473A) were associated with a higher AMD risk compared with the homozygous normal allele in the Turkish patients. The genetic factors in AMD may ultimately help identify high-risk populations, predict disease progression, and anticipate response to personalized therapy.

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