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Effect of *ARMS2* gene polymorphism on intravitreal ranibizumab treatment for neovascular age-related macular degeneration

H. Bardak[1], Y. Bardak[1], Y. Ercalik[1], E. Turkseven Kumral[1], S. Imamoglu[1], M. Gunay[2], H. Ozbas[3] and O. Bagci[3]

[1]Department of Ophthalmology,
Haydarapasa Numune Training and Research Hospital, Uskudar, Istanbul, Turkey
[2]Department of Ophthalmology, Trabzon Fatih State Hospital, Trabzon, Turkey
[3]Department of Medical Genetics, Suleyman Demirel University,
Faculty of Medicine, Isparta, Turkey

Corresponding author: H. Bardak
E-mail: handanbardak@yahoo.com.tr

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ABSTRACT. Age-related macular degeneration (AMD) is a leading cause of blindness in developed countries. The *ARMS2* gene has been found to be associated with AMD. Currently, intravitreal ranibizumab (IVR) treatment is one of the widely used treatments for neovascular AMD. The aim of this study was to investigate the association between the genotype of *ARMS2* rs10490924 polymorphism and IVR treatment responsiveness in patients with neovascular AMD. The study included 39 patients with advanced neovascular AMD (patient group) and 250 healthy individuals with exome sequencing data (control group). The

patient group was divided into three subgroups: GG (N = 10), TG (N = 14), and TT (N = 15). Before IVR treatment, all patients had intraretinal or subretinal fluid or both. They received three monthly IVR-injection treatments. One month after the third injection, the patients were evaluated as either "responders" or "non-responders" based on the presence or absence of intraretinal or subretinal fluid or both. The patient subgroups TG and TT had an 8.56- and 39-fold higher risk of AMD, respectively, than patient subgroup GG had. The allele frequency was 0.537 and 0.10 in the patient and control groups, respectively. Within the patient subgroup TT, there was a significant difference between the "responders" and "non-responders" (P = 0.025). In conclusion, in neovascular AMD patients undergoing IVR treatment, TT genotype tended to be a better predictor of good short-term treatment response, compared to the GG and TG genotypes. Further studies using confirmed genetic biomarkers for individualized optimal treatments are required.

Key words: Age-related macular degeneration;
Age-related maculopathy susceptibility protein 2 (*ARMS2*) gene;
Intravitreal ranibizumab; Polymorphism rs10490924;
Sequencing analysis

INTRODUCTION

Age-related macular degeneration (AMD) is one of the leading causes of blindness (Bourne et al., 2014; Wong et al., 2014; Zhang et al., 2016). The two main types of AMD are dry type AMD and neovascular type AMD. Neovascular AMD is characterized by the invasion of subretinal pigment epithelium and subretinal spaces by choroidal neovascularization, and geographic atrophy is typified by the degeneration of the choriocapillaris, Bruch's membrane, retinal pigment epithelium, and retina (Danis et al., 2015; Feeny et al., 2015; Schütze et al., 2015; Ferrington et al., 2016).

AMD is a complex disease associated with genetic and environmental risk factors. The predominant risk factors for AMD are age, family history, genetics, smoking, diet, and overexposure to sunlight (Gehrs et al., 2010; Mousavi and Armstrong, 2013).

AMD has been found to be associated with two adjacent genes on chromosome 10q26: age-related maculopathy susceptibility 2 (*ARMS2*) and high-temperature requirement factor H (*HTRA1*) (Gold et al., 2006; McKay et al., 2010). Between 50 and 60% of the disease etiology can be attributed to genetic variations that encode the complement factor H (*CFH*), *ARMS2*, and interleukin-8 (*IL-8*) (Cascella et al., 2014). Genes in the complement pathway, such as *CFH* (Edwards et al., 2005; Klein et al., 2005; Raychaudhuri et al., 2011), angiogenesis pathway, such as vascular endothelial growth factor (*VEGF*) (Yu et al., 2011), high-density lipoprotein metabolic pathway, such as cholesteryl ester transfer protein (*CETP*) (Liu et al., 2014), as well as the HtrA serine peptidase 1 (*HTRA1*) gene (Dewan et al., 2006) have been associated with AMD. In addition, complement components 3 and 9 have recently been found to be associated with AMD (Yanagisawa et al., 2011; Seddon et al., 2013).

Anti-VEGF agents such as bevacizumab and ranibizumab have provided significant enhancement in neovascular AMD treatment (Martin et al., 2011). Many AMD related single

nucleotide polymorphisms (SNPs) in different genes have been reported (Ding et al., 2009; Deangelis et al., 2011; Zhang et al 2015). Although the risks associated with these SNPs have been reported, the influence of these genetic variants on the response to therapy is still unclear (Lotery et al., 2013; Hagstrom et al., 2013, 2014). Through pharmacogenetic studies, the confirmed genetic biomarkers may allow for individualized treatment with optimized results.

The aim of this study was to investigate the association between the genotype of *ARMS2* gene rs10490924 polymorphism and the responsiveness of intravitreal ranibizumab (IVR) treatment in neovascular AMD patients. We hope that our study may be helpful for development of individualized optimal treatments.

MATERIAL AND METHODS

Ethics statement

This study was approved by the Local Ethics Committee of Suleyman Demirel University, School of Medicine. In addition, this study conforms to all the norms of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Patient recruitment

The patient database of the Haydarpasa Numune Training and Research Hospital, Department of Ophthalmology, Retina Clinic was searched for suitable patients who had the neovascular form of AMD and had undergone IVR treatment. Patients with a rapidly progressive, advanced, and neovascular instance of AMD were included in this study. Moreover, patients who received a diagnosis of systemic hypertension, diabetes mellitus type I or II, cardiovascular disease, or hyperlipidemia as well as those with smoking habits, history of ocular trauma, or occupational exposure to excessive ultraviolet radiation were excluded from this study. All the patients were unrelated to each other. Of the suitable individuals, a random sample of 39 was included in the patient group.

All participants underwent a complete eye examination, including best-corrected visual acuity (VA) measurement with the Snellen chart, slit-lamp biomicroscopy, indirect ophthalmoscopy, intraocular pressure measurement with the Goldmann applanation tonometer, fluorescein angiography (FFA) (Visucam 500, Carl Zeiss Meditec, Jena, Germany), and spectral-domain optical coherence tomography (OCT) (RTVue100; Optovue Inc., Fremont, CA, USA). In all patients, choroidal neovascularization was observed using FFA and OCT. Other causes of neovascularization, such as polypoidal choroidal vasculopathy and myopic choroidal neovascularization, were excluded.

DNA collection

The patient group included 39 patients with advanced neovascular AMD, while the control group consisted of 250 unrelated healthy subjects with exome sequencing data.

All peripheral blood samples were collected at the Haydarpasa Numune Training and Research Hospital, Department of Ophthalmology, where the proband was diagnosed with neovascular AMD based on clinical investigations. Genomic DNA was isolated from the peripheral blood using the Real Pure Spin kit (Real TM, Durviz, Spain) according to

the manufacturer protocol. We analyzed all coding exons of the *ARMS2* gene using next-generation sequencing (NGS).

Targeted NGS

The *ARMS2* gene-sequencing analysis was performed using the MiSeq NGS platform (Illumina, San Diego, CA, USA). The DNA samples were quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and used at a concentration of 50 ng/µL. Two exons of the *ARMS2* gene and their flanking splice site junctions were amplified with PCR primers designed using the PRIMER© - Primer Designer v 2.0 (Scientific & Educational Software program, Durham, NC, USA) software. The PCR products were validated using agarose gel electrophoresis. The primers for each individual were mixed to obtain PCR pools, which were purified using the NucleoFast® 96 PCR kit (Macherey-Nagel GmbH, Düren, Germany), quantified using the NanoDrop 1000, and then standardized to 0.2 ng/µL. The libraries were prepared by deploying the Nextera XT kit (Illumina Inc., San Diego, CA, USA), according to the manufacturer instructions.

Guanine (G)→thymine (T) base change at the 205th coding area, in the first exon of the *ARMS2* gene, led to A69S [alanine (A) - serine (S)] amino acid change, and is defined as rs10490924 polymorphism in the genetic databases.

The 39 patients in the patient group were divided into the following three subgroups based on their rs10490924 polymorphism genotype: patient subgroups GG, TG, and TT. Before IVR treatment, all patients had intraretinal or subretinal fluid or both. In accordance with the current guidelines, they received three monthly IVR injection treatments (Ho et al., 2014). One month after the third injection, the subjects were evaluated as either "responders" or "non-responders" based on the presence or absence of intraretinal or subretinal fluid or both. The "responder" group had no intraretinal or subretinal fluid or both, while the "nonresponder" group had these fluids. After three monthly injections, the subgroups were compared in terms of their IVR response.

Statistical analysis

Statistical analysis was performed using the SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). The strength of the association between rs10490924 polymorphism and AMD risk was assessed via logistic regression analysis. The nonparametric Wilcoxon signed rank test was used to evaluate the responsiveness of each subgroup to IVR treatment. In the analysis, an odds ratio (OR) with corresponding 95% confidence interval (CI) and $P < 0.05$ was considered statistically significant.

RESULTS

The patient subgroup GG (N = 10) included genetically normal patients with the GG allele at the rs10490924 polymorphic region. The individuals in subgroup TG (N = 14) had the TG allele at the rs10490924 polymorphic region, also known as the "heterozygote mutant", while subgroup TT (N = 15) included patients with the TT allele at the rs10490924 polymorphic region, which is also referred to as the "homozygote mutant".

Logistical regression analysis showed that the rs10490924 polymorphism was strongly

associated with AMD. The risk of AMD in the heterozygous risk allele (TG) and homozygous risk allele (TT) carriers was 8.56- and 39-fold higher than that in the homozygous carriers of the normal allele (GG; Table 1).

Table 1. Logistic regression analysis of rs 10490924 polymorphism in patient subgroups GG, TG, and TT.

Genotype	Case (N = 39)	Control (N = 250)	OR (95%CI)	P
GG (Normal)	10	208	1.0	
TG (Heterozygous mutant))	14	34	8.56 (3.52-20.83)	<0.0001
TT (Homozygous mutant)	15	8	39 (13.41-113.38)	<0.0001

OR = odds ratio; CI = Confidence interval; G = Guanine; T = Thymine.

The allele frequency in the patient and control groups was 0.537 and 0.10, respectively. The genome project minor allele frequency out of 1000 was 0.286 (Table 2).

Table 2. Allele frequency of cases and controls in 1000 Genome Project.

	Variation	Protein	dbSNP ID	MAF/1000 Genome Project	Allele frequency	Number of cases/controls	
						Homozygous	Heterozygous
Patient	c.205G>T	A69S	rs10490924	0.286	0.564	15	14
Control	c.205G>T	A69S	rs10490924	0.286	0.10	8	34

db SNP ID = The Single Nucleotide Polymorphism Database Identification; MAF = minor allele frequency.

The "responder" and "non-responder" patient distribution within the subgroups is shown in Table 3. There was a significant difference only between the "responder" and "non-responder" within the subgroup TT (P = 0.025; Table 3).

Table 3. Responder and non-responder patients in patient subgroups GG, TG, and TT.

		Patient subgroup GG (N = 10)	Patient subgroup TG (N = 14)	Patient subgroup TT (N = 15)
Before IVR treatment	Intra/Subretinal fluid	10 (100%)	14 (100%)	15 (100%)
1 month after 3 monthly IVR treatments	Responder	3 (30%)	3 (21.4%)	5 (33.3%)
	Nonresponder	7 (70%)	11 (78.6%)	10 (66.7%)
	P[a]	0.083	0.083	0.025*

[a]Wilcoxon Signed-Rank Test; *P < 0.05. G: guanine; T: thymine.

DISCUSSION

Previous studies have shown that the rs10490924 polymorphism, located in the *ARMS2* gene, has a strong association with AMD (Rivera et al., 2005; Ross et al., 2007; Fritsche et al., 2008; Tong et al., 2010; Fuse et al., 2011; Soysal et al., 2012; Tamura et al., 2012; Hirata et al., 2013). Similar results were observed in our study through logistical regression analysis. The risk of AMD in the heterozygous risk allele (TG) and homozygous risk allele (TT) carriers was 8.56-fold and 39-fold higher than that in the homozygous carriers of the normal allele. In rs10490924 polymorphism, alanine, the 69th amino acid of the ARMS2 protein, was substituted for serine as a missense mutation (Kanda et al., 2007). The allele frequencies for this polymorphism were 0.564 in the patient group, 0.010 in the control group, and 0.286 of 1000 in the genome project.

Soysal et al. (2012) studied the risk alleles of *ARMS2* gene polymorphism, rs10490924, in patients with AMD. They observed the following genotype distribution in their patients, 30.6% GG, 38.1% GT, and 31.3% TT; the T allele frequency observed was 0.504. Rivera et al. (2005) studied the same genetic region in 794 patients with AMD, and they reported a T allele

frequency of 0.417. Hirata et al. (2013) investigated the association between *ARMS2* gene rs10490924 polymorphism and AMD. They found that the T allele frequency was significantly higher in patients with AMD than in the controls (39.6% compared to 20.3%). The OR for AMD was 2.05 (95%CI = 1.13-3.71) for heterozygotes (TG) and 8.32 (95%CI = 2.30-45.99) for homozygotes (TT). Tong et al. (2010) studied the association of AMD with *ARMS2* gene rs10490924 G→T polymorphisms. In addition, they observed that the development of AMD risk for TT and TG genotypes were 7.512 and 2.353 times higher (in turn) than the risk of GG genotype. The results of our study were in agreement with the above results.

Currently, IVR treatment is the most widely used on-label treatment for neovascular AMD (Park et al., 2014). Although it is effective in most patients, some do not benefit from the treatment and 5-10% lose ≥15 letters despite the procedure (Rosenfeld et al., 2006; Brown et al., 2006; Martin et al., 2011; Chakravarthy et al., 2013). Moreover, IVR treatment is relatively expensive, uncomfortable, and may entail complications.

Genetic factors seem to be effective predictors of treatment responsiveness. There have been reports that *CFH* Y402H, *ARMS2* rs10490924, *HTRA1* rs11206038, and *VEGFA* polymorphisms are associated with responsiveness to ranibizumab treatment (Lee et al., 2009; Smailhodzic et al., 2012; Boltz et al., 2012; Chang et al., 2013). However, in the CATT and IVAN trials, which were multicentric randomized trials, the authors reported that there were no statistically significant associations between the genetic variants and anti-VEGF responsiveness (Hagstrom et al., 2013, 2014; Lotery et al., 2013). Therefore, the association of genetic factors with anti-VEGF treatment responsiveness in neovascular AMD remains unclear (Park et al., 2014).

The CATT results confirmed that anti-VEGF therapy is highly effective in the treatment of neovascular AMD (Martin et al., 2011). However, there is a wide range of clinical responses to therapy and variability in the number of injections required to achieve such responses. The mechanism underlying this heterogeneity in clinical response is unknown. The genetic factors and variations may be effective on the course of the disease (Hagstrom et al., 2013).

In this study, all the patients had intraretinal/subretinal fluid before IVR treatment. One month after the third IVR injection, there were 30% responders in patient subgroup GG, 21.4% responders in patient subgroup TG, and 33.3% responders in patient subgroup TT.

To date, several associative studies on the predictive role of rs10490924 in the treatment response of neovascular AMD have been reported. For example, Abedi et al. (2013) examined 17 SNPs in known AMD risk-associated genes. They found that the AA (homozygote risk) genotype at rs11200638-HTRA1 promoter SNPm and the TT (homozygote risk) genotype at rs10490924 (A69S) in LOC387715/*ARMS2* were both significantly associated with poorer VA outcomes following ranibizumab or bevacizumab injections. The main outcome measure of the above-mentioned study was VA, unlike the current study, in which we evaluated the anatomical success.

We found that the TT genotype at rs 10490924 responded pleasantly to IVR treatment in terms of anatomical improvement. In a previous study, Hagstrom et al. (2013) examined patients with neovascular AMD genotyped for SNPs rs1061170 (*CFH*), rs10490924 (*ARMS2*), rs11200638 (*HTRA1*), and rs2230199 (*C3*) to determine their response to ranibizumab or bevacizumab treatment. In their study, no statistically significant differences in response by genotype were identified for any of the clinical measures assessed. Specifically, there were no high-risk alleles that predicted final VA or changes in VA, the degree of anatomical response (presence of fluid on OCT or FA, retinal thickness, change in total foveal thickness, and change in lesion size), or the number of injections.

Meanwhile, Park et al. (2014) genotyped patients for 17 SNPs within 13 AMD-relevant genes. The minor allele homozygotes for *ARMS2* rs10490924 and *HTRA1* rs1100638 (GG genotypes for both) were associated with a greater central macular thickness reduction than the other genotypes, after anti-VEGF treatment. In their study, the patients used ranibizumab or bevacizumab or both; however, in our study, the patients used ranibizumab alone. Besides, the number of intravitreal injections was not similar to our study. It can be suggested that the characteristics of patients in that study might have caused these differences.

VA is a subjective measure influenced by the entire visual system, and it has been reported to be weakly correlated with retinal morphology, as evaluated using OCT (Moutray et al., 2008). We considered tomographic parameters as the treatment outcome measures that would reflect the anatomical changes after IVR therapy. This may be helpful in minimizing errors resulting from the use of vision alone as a measure of treatment response.

In our study, patients with different rs10490924 genotypes responded differently to IVR treatment. After a loading dose of three IVR injections, the number of eyes without intraretinal/subretinal fluid significantly changed only in the TT genotype (patient subgroup TT). Although the exact mechanism underlying the association between the TT genotype in rs10490924 and IVR treatment response is currently unknown, it can be hypothesized that this genotype may be associated with more effective downregulation of VEGF levels in the retina.

The limitations of this study are small sample size, retrospective nature, short follow-up time, and lack of evaluation of VA outcomes. In addition, all patients were recruited from a single center.

In conclusion, in patients with neovascular AMD undergoing IVR treatment, TT genotype tended to be a predictor of good short-term treatment response, compared to the GG and TG genotypes. Multicenter studies with larger series are necessary to reach a conclusion regarding individualized treatment regimens based on the patient genotype, with a view of achieving optimal treatment response in AMD.

Conflicts of interest

The authors declare no conflict of interest.

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