Genetics and Molecular Research

Next-generation sequencing analysis of the *ARMS2* gene in Turkish exudative age-related macular degeneration patients

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ABSTRACT. Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries. It is a complex disease with both genetic and environmental risk factors. To improve clinical management of this condition, it is important to develop risk assessment and prevention strategies for environmental influences, and establish a more effective treatment approach. The aim of the present study was to investigate age-related maculopathy susceptibility protein 2 (*ARMS2*) gene sequences among Turkish patients with exudative AMD. In addition to 39 advanced exudative AMD patients, 250 healthy individuals for whom exome sequencing data were available were included as a control group. Patients with a history of known environmental and

systemic AMD risk factors were excluded. Genomic DNA was isolated from peripheral blood and analyzed using next-generation sequencing. All coding exons of the *ARMS2* gene were assessed. Three different *ARMS2* sequence variations (rs10490923, rs2736911, and rs10490924) were identified in both the patient and control group. Within the control group, two further *ARMS2* gene variants (rs7088128 and rs36213074) were also detected. Logistic regression analysis revealed a relationship between the rs10490924 polymorphism and AMD in the Turkish population.

Key words: Age-related macular degeneration; *ARMS2* gene; Polymorphism; Sequencing analysis

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of progressive central vision loss and severe visual impairment in the elderly population (Wong et al., 2014). Two clinical forms are recognized: dry AMD and exudative AMD. The former is characterized by a thickening of Bruch's membrane, accumulation of drusen beneath the retinal pigment epithelium (RPE), and geographic atrophy, with loss of the RPE and photoreceptors in the macula. Exudative AMD, by contrast, is characterized by choroidal neovascularization (CNV), defined by the growth of new, fragile choroidal blood vessels, which penetrate Bruch's membrane, resulting in invasion of the retina with leaking vasculature (Ambati and Fowler, 2012; Veritti et al., 2012; van Lookeren Campagne et al., 2014; Kaszubski et al., 2016). The prevalence of AMD is 2.1% among individuals aged 40-49 years, increasing to 35% for those >80 years old. AMD affects nearly 8.7% of the elderly population (individuals > 55 years old) in developed countries (Ehrlich et al., 2008).

This disorder has a multifactorial etiology involving several genetic and environmental risk factors, including aging, smoking, family history of the disease, diet, oxidative stress, ultraviolet exposure, and hypertension. Inflammatory molecules (cytokines and chemokines), immune cells (macrophages), and complement proteins are also important in the development and progression of AMD (Friedman et al., 2004; Lim et al., 2012; Schwartz et al., 2012; Cascella et al., 2014; Sobrin and Seddon, 2014).

The gene *ARMS2* (also known as *LOC387715*) is located on chromosome 10q26.16 and encodes ARMS2, a small (11-kDa) protein specific to primates (Rivera et al., 2005). Sequence variants in chromosomal region 10q26, which harbors the corresponding gene, are associated with elevated AMD risk (Rivera et al., 2005; Dietzel et al., 2010; Miller, 2013). In close proximity, this locus also contains the gene encoding the serine protease HtrA serine peptidase 1 (*HTRA1*). The terminal exon of *ARMS2* is included within transcripts originating in an upstream gene (*PLEKHA1*), making for a complex situation (Kortvely and Ueffing, 2016). The results of genetic studies of AMD etiology have been inconsistent. Certain investigations have implicated *ARMS2* in this disease (Kanda et al., 2007; Fritsche et al., 2008), whereas others have challenged this conclusion (Yang et al., 2010; Friedrich et al., 2011). Kanda et al. (2007) and Fritsche et al. (2008) proposed that loss or mutation of *ARMS2* but not *HTRA1* is strongly associated with AMD. Therefore, we believe that further investigation of the *ARMS2* gene and the corresponding protein is warranted to determine their relationship with exudative AMD.

An association between AMD and genetic variations at two principal loci on chromosomes 1 and 10 has been confirmed (Dietzel et al., 2010; Miller, 2013). Zhou et al. (2015) identified two dry AMD-associated single nucleotide polymorphisms (SNPs) [rs7624556 (located on chromosome 3q24) and rs13119914 (4q34.3)] among the oldest individuals of the Han Chinese population. Moreover, Zhang et al. (2015) carried out a meta-analysis in which the rs2230199 polymorphism was found to contribute to the development of AMD. Genes associated with lipid metabolism and angiogenesis are involved in AMD pathogenesis and progression (Jo et al., 2015).

Following transfection of an *ARMS2* construct into mammalian cells, the encoded protein was observed to localize to the outer membranes of mitochondria (Kanda et al., 2007; Fritsche et al., 2008). Notably, a decrease in the number and size of mitochondria, loss of cristae, and reduced mitochondrial matrix density have been reported in AMD patients (Barron et al., 2001; Feher et al., 2006). The ARMS2 protein has also been detected in the cytoplasm and extracellular matrix (Wang et al., 2009; Kortvely et al., 2010). These results suggest that the *ARMS2* gene may play an important role in AMD pathogenesis.

Friedrich et al. (2011) and Yang et al. (2010) suggested that loss of *ARMS2* is insufficient to explain AMD susceptibility, as a nonsense mutation (R38X) in this gene resulting in a nonfunctional protein is included in an AMD-protective haplotype. Nevertheless, the functions of ARMS2 remain largely obscure (Kortvely and Ueffing, 2016). Relationships between risk and protective haplotypes and transcriptional and functional dysregulation of ARMS2 and HTRA1 proteins need to be elucidated.

Our results may help to identify new genetic markers of AMD. In the future, genetic testing using such markers may facilitate early AMD diagnosis and environmental risk factor assessment and prevention, and might even lead to more effective treatment. The aim of the present study was to investigate the *ARMS2* gene in Turkish patients with exudative AMD.

MATERIAL AND METHODS

Ethics statement

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

Patient recruitment

The patient database of the Haydarpaşa Numune Hospital Ophthalmology Department Retina Clinic was searched for suitable patients with the neovascular (exudative) form of AMD having undergone intravitreal anti-VEGF injection treatment. To be included, patients had to exhibit rapidly progressive, advanced neovascular AMD. Patients diagnosed with systemic hypertension, diabetes mellitus type I or II, cardiovascular disease, or hyperlipidemia were excluded. Patients with a smoking habit, a history of ocular trauma, or jobs involving excessive ultraviolet exposure were also excluded. All patients were unrelated. Of the eligible individuals, 39 were randomly selected as the patient group.

All patients underwent a complete eye examination, including best corrected visual acuity measurement using a Snellen chart, slit-lamp biomicroscopy, indirect ophthalmoscopy,

intraocular pressure measurement by Goldmann applanation tonometry, fluorescein angiography (FFA) (Visucam 500; Carl Zeiss Meditec, Jena, Germany), and spectral-domain optical coherence tomography (OCT) (RTVue-100; Optovue Inc., Fremont, CA, USA). CNV was observed in all examined eyes by FFA and OCT. Patients with CNV resulting from conditions other than AMD were excluded.

DNA collection

The study group included 39 advanced exudative AMD patients. The control group consisted of 250 unrelated healthy subjects for whom exome sequencing data were available. All peripheral blood samples were collected at the Haydarpaşa Numune Training and Research Hospital Department of Ophthalmology, where the patients were diagnosed as having exudative AMD based on clinical assessment. Genomic DNA was isolated from peripheral blood samples using a Real Pure Spin kit (Durviz, Valencia, Spain) following the manufacturer protocol. All coding exons of the *ARMS2* gene were analyzed by next-generation sequencing (NGS).

Targeted NGS

ARMS2 gene sequencing analysis was performed using the MiSeq NGS platform (Illumina, San Diego, CA, USA). 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. The two exons of the *ARMS2* gene and their flanking splice site junctions were amplified using polymerase chain reaction (PCR) primers designed with the PRIMER© - Primer Designer v.2.0 software (Scientific & Educational Software, Denver, CO, USA). PCRs were validated by agarose gel electrophoresis. PCR products for each individual were mixed to obtain PCR pools, which were then purified using a NucleoFast® 96 PCR kit (Macherey-Nagel GmbH, Düren, Germany). Following quantification with a NanoDrop 1000, the concentration of each pool was adjusted to 0.2 ng/μL. The libraries were prepared with a Nextera XT kit according to the manufacturer instructions.

Statistical analysis

Statistical analysis was performed using the SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). The strength of association between the three polymorphisms (rs10490923, rs2736911, and rs10490924) and AMD risk was assessed by logistic regression analysis. Odds ratios with corresponding 95% confidence intervals were calculated, with P values <0.05 being considered statistically significant.

RESULTS

The patient group consisted of 19 women (48.7%) and 20 men (51.3%) with a mean age of 75.7 ± 7.8 years. Three different *ARMS2* gene sequence variations (rs10490923, rs2736911, and rs10490924) were detected in both the patient and control group. In addition, two further *ARMS2* variants (rs7088128 and rs362130074) were identified among the control subjects (Table 1).

Table 1. Genetic variations detected in patient and control groups.

Group	Variation	Protein	dbSNP ID	MAF/1000 Genomes Project	Allele frequency	Number of cases/controls	
						Homozygous	Heterozygous
Case	c.8G>A	R3H	rs10490923	0.075	0.090	1	5
	c.112C>A	R38R	rs2736911	0.114	0.077	1	4
	c.205G>T	A69S	rs10490924	0.286	0.564	15	14
Control	c.8G>A	R3H	rs10490923	0.075	0.086	4	35
	c.112C>A	R38R	rs2736911	0.114	0.072	2	32
	c.205G>T	A69S	rs10490924	0.286	0.10	8	34
	IVS1-13A>G	Intronic	rs7088128	0.095	0.022	0	11
	c.298-26T>C	Intronic	rs36213074	0.074	0.064	2	28

dbSNP ID = single nucleotide polymorphism database identification; MAF = global minor allele frequency.

The rs10490923 polymorphism comprises a missense mutation resulting in the substitution of arginine with histidine at the 3rd position of the ARMS2 amino acid sequence. Frequencies for this polymorphism were 0.090, 0.086, and 0.075 among patients, controls, and in the 1000 Genomes Project dataset, respectively.

The 38th amino acid of the ARMS2 protein is arginine, encoded by the codon CGG. The rs2736911 SNP affects the first base, replacing cytosine with adenine, resulting in the trinucleotide AGG. This encodes the same amino acid (arginine), and therefore represents a synonymous or silent mutation. This polymorphism was present at frequencies of 0.077, 0.072, and 0.114 in the patient and control group, and 1000 Genomes Project database, respectively.

The rs10490924 variant is a missense mutation causing alanine, the 69th amino acid of the ARMS2 protein, to be substituted with serine. Its frequencies were found to be 0.564, 0.10, and 0.286 among patients, controls, and the 1000 Genomes Project data, respectively. Logistic regression analysis revealed a significant difference ($P < 0.0001$), identifying an association between the rs10490924 polymorphism and AMD (Table 2).

Table 2. Logistic regression analysis of genetic variations detected in patient and control groups.

dbSNP ID	Cases (N = 39)	Controls (N = 250)	OR (95%CI)	P
rs10490923				
AA	33	211	1.0	
GA	5	35	0.91 (0.33-2.49)	0.86
GG	1	4	1.59 (0.17-14.74)	0.67
rs2736911				
AA	34	216	1.0	
AC	4	32	0.79 (0.26-2.38)	0.68
CC	1	2	3.17 (0.28-35.99)	0.35
rs10490924				
GG	10	208	1.0	
TG	14	34	8.56 (3.52-20.83)	<0.0001
TT	15	8	39 (13.41-113.38)	<0.0001

dbSNP ID = single nucleotide polymorphism database identification; OR = odds ratio; CI = confidence interval.

DISCUSSION

Certain reports have suggested that genes in chromosome regions 1q (1q25-31) and 10q (10q26) play roles in AMD pathogenesis. It has been reported that three genes in particular, *PLEKHA1*, *LOC387715*/*ARMS2*, and *HTRA1*, on chromosome 10q26, are highly likely to be involved in this disease (Majewski et al., 2003; Fisher et al., 2005).

A relationship between AMD and the *LOC387715*/*ARMS2* and *HTRA1* genes has

been established in a linkage analysis (Jakobsdottir et al., 2005). An association has also been demonstrated between the *ARMS2* gene and both exudative and dry AMD forms (Fisher et al., 2005; Kanda et al., 2007). Kanda et al. (2007) assessed the link between AMD and 45 different SNPs of the *LOC387715*/*ARMS2*, *HTRA1*, and *PLEKHA1* genes, finding only the A69S (rs10490924) variant on chromosome 10q26 to be significantly implicated. These authors also showed this *LOC387715*/*ARMS2* gene polymorphism to be related to mitochondria, suggesting that this might explain the apparent roles of aging and oxidative stress in AMD pathogenesis. Ross et al. (2007) reported a significant association between AMD and heterozygous and homozygous carriers of rs10490924 risk alleles. Moreover, this polymorphism exhibits a stronger relationship with exudative than dry AMD (Jakobsdottir et al., 2005; Allikmets and Dean, 2008; Wang et al., 2008).

Fritsche et al. (2008) first identified a polymorphism composed of a 443-bp deletion followed by a 54-bp insertion (del443ins54) in the 3'-untranslated region of *ARMS2* in 2008. A molecular study demonstrated that this variation generates an unstable mRNA transcript subject to rapid degradation, thus compromising translation of the corresponding protein (Wang et al., 2009). Genotyping of the del443ins54 polymorphism in the Italian population revealed a significant association with AMD susceptibility (Ricci et al., 2009).

Successive case-control and genome-wide association studies have implicated the *ARMS2* SNP rs10490924 (G/T) in AMD (Fritsche et al., 2008). This variant is responsible for the nonsynonymous amino acid change A69S, which increases the risk of developing AMD for individuals homozygous for the T allele by 7.6-fold compared to heterozygotes. Interestingly, the del443ins54 and rs10490924 variations have been found to be in strong linkage disequilibrium, simplifying the simultaneous genetic and molecular characterization of these two risk variants, both of which are present at the 10q26.13 locus (Seddon et al., 2010).

Furthermore, the presence of risk alleles at this locus has been correlated with elevated levels of C-reactive protein (CRP) in subjects with no evidence of AMD compared to individuals carrying the wild-type genotype (Yasuma et al., 2010). The relationship observed between *ARMS2* risk variants and high serum CRP levels suggests the involvement of gene polymorphisms in inflammation, and perhaps AMD development and progression (Seddon et al., 2010). Supporting this hypothesis, a possible relationship between *ARMS2* mRNA expression and certain proinflammatory molecules (IL-6, IL-8, TNF-α, C3, and C5) has been described, outlining a potential AMD pathogenesis mechanism (Seddon et al., 2010; Zeng et al., 2013).

ARMS2 gene polymorphism has been reported to be a significant AMD risk factor in the Spanish population (Brión et al., 2011). In addition, Cruz-González et al. (2014) identified a significant association between rs10490923 and the risk of developing AMD among Spanish individuals. Gotoh et al. (2009) determined that Japanese subjects heterozygous for the rs10490924 polymorphism are at a three-fold higher risk of AMD, but did not detect the rs10490923 variant in the population examined. Moreover, Bergeron-Sawitzke et al. (2009) reported that the rs10490924 SNP in exon 1 of *ARMS2* is significantly associated with AMD.

Fritsche et al. (2008) suggested that the 372-815 del443ins54 variation in the *ARMS2* gene could be a functional variant. They have also been shown that this *ARMS2* polymorphism significantly increases disease risk for both heterozygous and homozygous carriers, and that *ARMS2* is not expressed by the latter (Barreau et al., 2006; Garneau et al., 2007).

In the present study, we detected three SNPs (rs10490923, rs2736911, and rs10490924) among the AMD patients investigated. We determined that only rs10490924 was significantly associated with this disease based on logistic regression analysis. In addition, we showed that

AMD risk was 8.56- and 39-fold higher for heterozygous and homozygous risk allele carriers, respectively, compared to individuals homozygous for the normal allele.

Two intronic variations (rs7088128 and rs36213074) found in the control group but not the patient group may exert a protective effect against AMD. However, the allele frequencies of these two variants in our study group were similar to those in the MAF/1000 Genomes Project data; therefore, this possibility seems unlikely.

The most widely accepted hypothesis regarding the influence of genetic variations on biological mechanisms related to disease involves aging-associated mitochondrial pathways. Neurodegenerative conditions linked to aging, such as Alzheimer's and Parkinson's disease, are also associated with mitochondria. Mitochondrial dysfunction resulting from aging may lead to compromised energy metabolism, accumulation of mitochondrial DNA mutations, and subsequent activation of apoptotic pathways (Lin and Beal, 2006; McBride et al., 2006). Compared to healthy individuals, in the retinas AMD patients, mitochondrial number and size are decreased, cristae are lost, and mitochondrial DNA mutations accumulate (Veritti et al., 2012; Kaszubski et al., 2016).

In the present study, three different *ARMS2* gene sequence variations (rs10490923, rs2736911, and rs10490924) were detected in both the patient and control group. Among control subjects, two further *ARMS2* variants (rs7088128 and rs362130074) were also identified. Logistic regression analysis of the sequence variations observed revealed a relationship between the rs10490924 polymorphism and AMD in the Turkish population. However, our study was limited by possible patient selection bias and the small number of patients involved. Further molecular studies are necessary to evaluate the role of this genetic variation in AMD pathogenesis.

Conflicts of interest

The authors declare no conflict of interest.

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