

# The effect of *CFH* polymorphisms on the response to the treatment of age-related macular degeneration (AMD) with intravitreal ranibizumab

Ozlem Dikmetas, Sibel Kadayıfcılar, Bora Eldem

*Hacettepe University School of Medicine, Department of Ophthalmology*

**Purpose:** The purpose of this study is to evaluate the effect of complement factor H (*CFH*) Y402H CC and TT polymorphisms on treatment response to intravitreal ranibizumab injection in patients with wet age-related macular degeneration (AMD).

**Methods:** One hundred ninety-three patients with choroidal neovascularization (CNV) secondary to AMD who were monitored for at least 6 months of follow-up, and with at least three ranibizumab injections, were included in the study. At the final examination, an increase in visual acuity (VA) of five letters or more compared to the initial VA was regarded as a good response, and a decrease in VA of five letters or more compared to the initial VA was evaluated as a poor response. A genetic examination was performed with a PCR melting curve analysis. In the statistical evaluation, SPSS version 18 software was used.

**Results:** The mean age of the patients was 71.01 (55–86) years, the mean follow-up was 13.34 (6–36) months, and the mean number of injections was 4.02 (3–15). There were 96 patients in the good response group (Group 1) and 97 patients in the poor response group (Group 2). The initial VA in Group 1 was 41.34 (10–64) letters, the initial central macular thickness (CMT) was 213.40 (126–494)  $\mu\text{m}$ , and the initial lesion width was 3760 (1430–6430)  $\mu\text{m}$ . The initial VA in Group 2 was 52.89 (26–82) letters, the initial CMT was 257.60 (115–882)  $\mu\text{m}$ , and the initial lesion width was 4460 (1000–7650)  $\mu\text{m}$ . There was no statistically significant difference between the two groups in terms of the initial VA and CMT ( $p=0.094$ ,  $p=0.083$ ). However, there was a statistically significant difference between the groups in the width of the initial lesion ( $p=0.003$ ). In Group 1, 15 CC, 30 TT, and 51 TC alleles were found, and in Group 2, 49 CC, two TT, and 46 TC alleles were found, and the distribution was significantly different between the two groups ( $p=0.012$ ). The change in the distribution of genotypes was not associated with either the lesion size or VA ( $p=0.841$ ). Fibrosis developed in 12 patients who were all poor responders.

**Conclusions:** *CFH* Y402H CC accompanied a poor response, and TT accompanied a good response in this series of patients with AMD undergoing ranibizumab therapy.

Age-related macular degeneration (AMD) is the most common cause of central loss of vision among the population aged 65 years or older in developed countries [1]. There are two basic forms of AMD: neovascular (wet) and non-neovascular (dry). The neovascular form accounts for 10% of all cases. Dry AMD is characterized by alterations in the retinal pigment epithelium (RPE), atrophy, and drusen. Wet AMD is characterized by choroidal neovascularization (CNV) [2]. In the United States, severe AMD in at least one eye affects 1.75 million people over 40 years of age, and this number is estimated to increase by 50% in 2020, placing 7 million people at the risk of AMD [3]. No comprehensive data on the prevalence of AMD exists in our country, Turkey. Worldwide, 500,000 new cases of neovascular AMD occur each year. Angiogenesis triggered by unknown reasons results

in CNV in the pathogenesis of wet AMD, which accounts for 90% of the blindness caused by the disease. Advanced age and smoking are the most important proven risk factors. Apart from these established factors, genetics, race, gender, socioeconomic status, refractive errors, obesity, vitamins, systemic disorders, and hormonal factors are also thought to be involved [4]. The most important component of angiogenesis is vascular endothelial growth factor (VEGF)-A, which has nine isoforms depending on the number of amino acids contained. VEGF acts to increase vascular permeability and to induce endothelial fenestration. Increased vascular permeability results in interstitial protein accumulation and creates a suitable environment for angiogenesis. Increased levels of VEGF also result in the development of macular edema. VEGF is the principal angiogenic substance responsible for the development of neovascularization in age-related macular degeneration, as well as in diabetic retinopathy. In recent years, the suppression of VEGF by VEGF-directed antibodies has become one of the most common therapeutic

Correspondence to: Sibel Kadayıfcılar, Hacettepe University, Department of Ophthalmology, Ankara, Turkey; Phone: 90-312-3051777; FAX: 90-312-3094101; email: sibelkadayifcilar@yahoo.com

options in managing retinal neovascularization, CNV, and macular edema [4]. Genetic and environmental risk factors have an important place in the etiopathogenesis of AMD. Genetic factors are thought to be present in up to 71% of cases whereas 29% of cases with AMD are attributed to environmental factors [5,6]. Many different genes are thought to contribute to total genetic risk. In the last decade, research has focused on the genetic component of AMD. The reason for the shift in focus toward genetic analysis is that studies have found mutations and polymorphisms that could affect the life-long risk of developing AMD. However, it is more challenging to reveal genetic factors in the older age group because the condition by its nature becomes more common with advancing age. This is because the focus here is only one generation, and it may not be possible to detect the condition in parents and children [7,8]. The complement system, which is part of the immune system and plays an important role in inflammation, is also involved in the pathogenesis of AMD [9,10]. C3, C5, and C5b-9 complex, the components of the complement cascade, have been detected in drusen and in the surrounding space. Complement factor H (CFH) protein, one of the molecules of the complement system, is an important regulator of the alternative pathway of complement activation. This molecule is required to limit complement activation and possesses anti-inflammatory effects. Genetic variations of *CFH* are known to increase the risk of inflammatory disease. In the studies conducted in Europe and the United States, a missense single nucleotide polymorphism (SNP) of *CFH* has emerged as a risk factor for developing AMD; however, a study in Japan did not implicate this polymorphism as a risk factor [9,11,12]. This polymorphism produces different results in different countries, and limited studies conducted in cases with AMD in Turkey have yielded comparable results with the other countries for the ratio of two polymorphisms (CC and TT) of the *CFH* gene [12]. These studies focused only on the frequency of polymorphisms within the population and did not study their effect on the response to therapy. Studies in other countries have investigated the effects of genetic polymorphisms on the response to therapy with intravitreal bevacizumab and ranibizumab administration; the *CFH* Y402H CC polymorphism has been associated with poor response and the TT polymorphism with good response, and researchers have noted an improvement in visual acuity after therapy in this group of patients. The purpose of this study was to evaluate the effect of *CFH* Y402H rs1061170 CC and TT polymorphisms on treatment response to intravitreal ranibizumab injection in Turkish patients with a diagnosis of wet AMD.

## METHODS

Following institutional ethics board approval (LUT 11/10 dated 21.02.2011), peripheral blood samples from 193 patients who had applied to Hacettepe University School of Medicine, Department of Ophthalmology's Retina Unit for intravitreal ranibizumab treatment for neovascular AMD between May 2011 and May 2012 were collected and examined. Only patients with CNV development secondary to AMD and with at least 6 months of follow-up were included in the study. The other inclusion criteria were being aged 55 years and older and the application of at least three injections. The exclusion criteria were consanguinity, the presence of other eye diseases, trauma, angioid streaks, development of CNV secondary to inflammatory diseases, follow-up of less than 6 months, more than 50% of the lesion being hemorrhagic, and the presence of fibrosis, atrophy, or pigment epithelial detachment (PED) in more than 50% of the lesion and involving the fovea. None of the patients had positive family history for wet AMD. In the final examination, an increase in VA of five letters or more compared to the initial examination was evaluated as a good response, and a decrease in VA of five letters or more compared to the initial examination was evaluated as a bad response. Good response and bad response groups had two subunits; an increase in VA of 15 letters or more was very good, an increase in VA of five to 14 letters good, a decrease in VA of 15 letters or more very bad, and a decrease in VA of five to 14 letters bad response. We did not include patients with stable, unchanged VA within five letters.

The patients' medical history included smoking, presence of hypertension, cardiovascular diseases, and current and past medication. All patients underwent a complete ophthalmological examination before the injection and subsequent monthly examinations. Anterior segment examinations were performed with slit-lamp microscopy, and dilated fundus examinations were performed with a 90D lens. The VA of all cases was assessed with Early Treatment of Diabetic Retinopathy Study (ETDRS) charts. Central macular thickness was evaluated with Stratus OCT (Zeiss, Oberkochen, Germany). Lesion width was evaluated with fundus fluorescein angiography (FFA). The lesion width was evaluated from 30° images taken during FFA. The images were manually traced with the computer mouse to delineate the total lesion area including occult and classic CNV components, serous PED, and any hemorrhage dense enough to cover underlying fluorescence by the same examiner (SK). The greatest linear diameter of the lesion was then calculated with Visupac imaging software (Carl Zeiss Meditec AG, Jena, Germany) automatically. Patients were monitored at monthly intervals

after the intravitreal ranibizumab injection. Repeat injection criteria were determined as at least a 100  $\mu\text{m}$  increase in central thickness in OCT, recurrent fluid or novel fluid accumulation in OCT, together with clinical findings of a VA loss of five letters or more, not associated with increased cataract or atrophy, and recent macular hemorrhage.

The study was conducted according to the Declaration of Helsinki Principles. All patients were divided into two groups as good and bad responders, as previously defined, and a 10 ml venous blood sample was collected from an antecubital vein into EDTA tubes, after obtaining informed written consent for the study. The genomic DNA was extracted from whole blood with High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) and then samples were preserved at  $-20\text{ }^{\circ}\text{C}$  in the deep freeze (Liebherr, Berlin, Germany) until analysis. Five  $\mu\text{l}$  (50 ng) of each genomic DNA was mixed with 15  $\mu\text{l}$  of reaction mixture containing 1.0  $\mu\text{l}$  of reagent mix, 2.0  $\mu\text{l}$  of Fast-Start DNA master (Roche Diagnostics), and 1.6  $\mu\text{l}$  of  $\text{MgCl}_2$ . The genetic analysis was carried out with a [rs1061170](#) LightSNiP (TIB Molbiol, Berlin, Germany) commercial kit, using a real-time PCR device, LightCycler480 (Roche, Mannheim, Germany), automatically. This method was reported to be accurate, homogenous and rapid method for CFH phenotyping previously [13]. The LightSNiP kit was designed as an individual SNP base by TIB MOLBIOL (ATTTggAAAATggATATAATCAAAAT C/T A TggAAgAAAgtTTgTACAgggTAA). Amplification and melting curve analysis was performed through the 465-510 detection format of the LightCycler 480 device with the following parameters: Detection Format, Pre-Incubation  $-95\text{ }^{\circ}\text{C}$  3 min, Amplification/Quantification ( $95\text{ }^{\circ}\text{C}$  10 s,  $60\text{ }^{\circ}\text{C}$  10 s,  $72\text{ }^{\circ}\text{C}$  15 s w/single acquisition; 45 cycles), High Resolution Melting ( $95\text{ }^{\circ}\text{C}$  30 s,  $40\text{ }^{\circ}\text{C}$  2 min,  $75\text{ }^{\circ}\text{C}$  1 s,  $90\text{ }^{\circ}\text{C}$  w/25 acquisitions per  $^{\circ}\text{C}$ ). Fluorescence intensities of melting curves were normalized within the software. The samples were evaluated as CFH Y402H [rs1061170](#) TT and CC mutant, heterozygote mutant or wild type.

**Statistics:** In the statistical evaluation SPSS 18 software was used. For descriptive purposes box plot graphs, mean, median, range and standard deviation were used to summarize the data. Chi-square test, Student's t test, and Mann-Whitney U-test were used to compare the two groups. For three group comparisons chi-square test, analysis of variance, and Kruskal-Wallis test were used. Hardy-Weinberg equilibrium was calculated.

## RESULTS

Ninety patients were female (46.63%), and 103 were male (53.37%); the mean age was 71.01 (55–86) years. When the patient distribution between the two groups was evaluated, there were 96 patients in the good response group, comprising 49.74% of all patients, and there were 97 patients in the bad response group, comprising 50.25% of all patients. There were 43 women and 53 men in the good response group, and 47 women and 50 men in the bad response group. No statistically significant difference was found between these two groups regarding gender ( $p=0.72$ ). The mean age of patients in the good response group was  $71.53\pm 7.44$  (55–85) years, and the mean age of patients in the bad response group was  $70.44\pm 7.04$  (55–86) years. No statistically significant difference was found between these two groups ( $p=0.30$ ) in terms of age. When the groups are compared regarding smoking, 22.68% of the good response group and 18.75% of the bad response group were smokers. The difference was not statistically significant ( $p=0.87$ ). Hypertension was detected in 9.37% of patients in the good response group and 13.40% of patients in the bad response group, and the difference was not statistically significant ( $p=0.74$ ). According to these data, the good and bad response groups had similar demographic features.

The two groups were assessed and compared in terms of clinical features before and after intravitreal ranibizumab injection. The initial VA with ETDRS was determined as 41.34 (10–64) letters in the good response group and as 52.89 (26–82) in the bad response group. No significant difference was found between the two groups regarding the initial VA ( $p=0.09$ ). The final VA was 57.83 (28–89) letters in the good response group and 36.29 (8–59) letters in the bad response group, and a statistically significant difference was found between the two groups ( $p=0.018$ ). There was a marked response to treatment in the good response group, and VA increased significantly. The initial macular thickness was determined with OCT, and no significant difference was found ( $p=0.08$ ; Table 1). When the initial lesion width was compared with FFA, a significant difference was detected between the two groups ( $p=0.003$ ). The initial lesion was significantly larger in the bad response group (Table 1). The number of injections and follow-up periods were also compared between the two groups. The mean number of injections was 4.02 (3–15) in the good response group and 4.40 (3–16) in the bad response group, and no significant difference was found between the two groups ( $p=0.654$ ). The mean follow-up period was 13.34 (6–36) months. The number of patients under the 6–12 month follow-up were 55/96 (57.29%), 13–24 months 28/96 (29.16%), and 25–36

months 13/96 (13.53%) in the good response group and 13.28 (6–43) months in the bad response group. The number of patients under the 6–12 month follow-up were 57/97 (58.76%), 13–24 months 28/97 (28.86%), 25–36 months 10/97 (10.30%), and more than 37 months 2/97 (2.06%). No significant difference was found between the two groups ( $p=0.522$ ; Table 1). When the varying letter values during the treatment period were evaluated, there were at least a five letter and at most a 61 letter increase in the good response group. In the bad response group, a decrease of at least five letters and at most 49 letters was observed. When the genotype distribution in peripheral blood was evaluated in both groups, 15 patients had the CC genotype, 30 patients had the TT genotype, and 51 patients had the TC genotype in the good response group, and 49 patients had the CC genotype, two patients had the TT genotype, 46 patients had the TC genotype in the bad response group. Our study population conformed to the Hardy–Weinberg equilibrium. The genotype difference between the two groups was significant, and the CC genotype in the bad response group and the TT genotype in the good response group were significantly high ( $p=0.012$ ; Table 2). The VA change according to all three genotype groups was compared. In the good response group and in the bad response group, the changes were not significant among the three genotype groups ( $p=0.5$  and  $p=0.573$ , respectively; Table 3). In the good response group, the number of patients with an increase in VA of 15 letters or more (the very good response group) and with fewer than 15 letters was compared according to all three genotype groups. An increase of fewer than 15 letters was found in 14 patients (46.7%) for the TT genotype, in 27 patients (52.9%) for the TC genotype, and in four patients (26.7%) for the CC genotype. An increase of more than 15 letters was found in 16 patients (53.3%) for the TT genotype, in 24 patients (47.1%) for the TC genotype, and in 11 patients (73.3%) for the CC genotype; no significant difference was found among the three genotype groups ( $p=0.200$ ). In the bad response group, the number of patients with a decrease in VA of 15 letters or more (the very bad

response group) and with fewer than 15 letters was compared according to all three genotype groups. An increase of fewer than 15 letters was found in no patients (0%) in the TT genotype, in 18 patients (39.1%) in the TC genotype, and in 16 patients (32.7%) in the CC genotype, while a decrease of more than 15 letters was found in two patients (100%) in the TT genotype, in 28 patients (60.9%) in the TC genotype, and in 33 patients (67.3%) in the CC genotype. No significant difference was found among the three genotype groups ( $p=0.349$ ). When the distribution of initial lesion width according to all three genotype groups in the good and bad response groups was evaluated, the initial lesion width in the good response group was  $4.07\pm 1.82$  in the CC genotype,  $3.91\pm 2.96$  in the TT genotype, and  $3.58\pm 1.57$  in the TC genotype, and no significant difference was found among the three genotype groups ( $p=0.841$ ). In the bad response group, the initial lesion width was  $4.40\pm 1.49$  in the CC genotype,  $4.90\pm 1.55$  in the TT genotype, and  $4.48\pm 1.55$  in the TC genotype, and no significant difference was found among the three genotype groups ( $p=0.455$ ). The central macular thickness (CMT) change was compared in the good and bad response groups according to all three genotype groups (Table 4 and Table 5). No systemic side effect was observed during the study period; however, PED in eight, fibrosis in 12, and hemorrhage in six cases developed in the bad response group.

## DISCUSSION

The current treatment for wet AMD involves anti-VEGF therapy. However, anti-VEGF therapy does not produce the same result in all patients. Factors affecting the response to anti-VEGF therapy include injection regimen, baseline VA and lesion size, presence of subretinal hemorrhage, PED, vitreomacular adhesion, cystoid macular edema, and the presence of thick subretinal tissue [2,4,5,12]. Other risk factors affecting response to anti-VEGF therapy include age, smoking, race, genetics, follow-up time, antioxidant use, blood HDL levels, and concomitant cardiovascular disorders

TABLE 1. CLINICAL FEATURES

Variables	Good response average (Interval; n=96)	Bad response average (Interval; n=97)	p value
Initial VA (Letters)	41.34 (10–64)	52.89 (26–82)	0.094
Final VA (Letters)	57.83(28–89)	36.29 (8–59)	<b>0.018</b>
Initial CMT (µm)	213.40 (126–494)	257.60 (115–882)	0.083
Initial lesion width (Average; mm)	3.76 (1.43–6.43)	4.46 (1.00–7.65)	<b>0.003</b>
Number of injections	4.02 (3–15)	4.40 (3–16)	0.654
Follow up period (Months)	13.34 (6–36)	13.28 (6–43)	0.522

**TABLE 2. NUMERICAL DISTRIBUTION OF CC, TT, AND TC GENOTYPES IN THE GOOD RESPONSE AND BAD RESPONSE GROUPS**

Group	CC (n)	TT (n)	TC (n)	p
Good response group	15	30	51	<b>0.012</b>
Bad response group	49	2	46	

**TABLE 3. DISTRIBUTION OF MINIMUM AND MAXIMUM LETTER CHANGES ACCORDING TO THE THREE GENOTYPE GROUPS IN THE BAD RESPONSE GROUP**

Change	CC	TT	TC	p
Minimal letter change	-6	-15	-5	0.573
Maximal letter change	-49	-25	-31	
Mean	-17.71	-20	-15.65	

[4,12,14]. Our study did not reveal any difference between the groups in terms of VA at baseline. However, poorer response was observed in patients with larger lesions. Similar to the current study, MARINA and ANCHOR, previous multicenter studies with ranibizumab, found a poor response among patients with large lesions and low VA at baseline [15,16]. The same studies disclosed age was an important factor influencing treatment outcomes and younger patients had a better rate of response to therapy [15,16]. The patients in the current study were of similar ages, and therefore, no such effect was observed. Our groups did not differ regarding smoking and presence of hypertension. Since PED and presence of vitreomacular adhesions were previously shown to be associated with poor response [17,18], patients with these characteristics were excluded in our study. Follow-up time is one of the most important factors for visual prognosis [14] but it was not different between our study groups.

Previous studies disclosed genetic parameters as other important factors affecting response to therapy. *ApoE*, *CFH* Y402H, *PLEKHA1/ARMS2(LOC38771)/HtrA1*, complement factor B, complement component 2, VEGF, ABCA4, excision-repair cross-complementing group 6 (*ERCC6*), and fibulin 5 (*FBLN5*) genes are the most important markers in AMD [6]. *CFH* exerts its effect on the alternative complement pathway, and normal *CFH* inhibits C3 activation. The *CFH* variant induces uncontrolled complement activation and thus results in abnormal inflammation [9]. It has been long known that the prevalence of AMD varies among different ethnic groups. In addition, AMD phenotypes are substantially heterogeneous between these groups. Although different results have been obtained from different populations, the *CFH* Y402H polymorphism has been important for developing AMD in France, Germany, China, Australia, and England but not in Japan [9,11]. In a study by Souied et al. in France, the frequency of the C allele was high, as in our study, and CC and CT polymorphisms were significantly more

**TABLE 4. DISTRIBUTION OF MINIMUM AND MAXIMUM CENTRAL MACULAR THICKNESS (CMT) CHANGES WITH OCT IN THE GOOD RESPONSE GROUP.**

Allele	Minimal CMT decrease (µm)	Maximal CMT decrease (µm)	p
TC	-233	-246	0.380
CC	-68	-125	
TT	-168	-295	

**TABLE 5. DISTRIBUTION OF MINIMUM AND MAXIMUM CENTRAL MACULAR THICKNESS (CMT) CHANGES WITH OCT IN THE BAD RESPONSE GROUP.**

Allele	Minimal CMT decrease (µm)	Maximal CMT decrease (µm)	p
TC	-269	-259	<b>0.143</b>
CC	-166	-148	
TT	-4	-12	

frequent among patients with wet AMD, with no significant difference between familial and sporadic cases [19]. Familial cases were not included in our study. Studies evaluating the distribution of the *CFH* Y402H polymorphism in AMD are scarce in Turkey. Soysal et al. studied *CFH* Y402H and *LOC387715* A69S polymorphisms in 147 patients with AMD and 105 controls; CC and TC polymorphisms were significantly higher in the AMD group, a finding similar to that in other European populations. The TT polymorphism was higher in the control group, the GG and GT polymorphisms were higher in the *LOC387715* control group, and the TT polymorphisms were significantly higher in the AMD group [20].

In our study, poor response was observed in patients with larger lesions, but no difference was found among the alleles. Seitsonen et al. investigated the association of *CFH* Y402H polymorphism with lesion size; the mean lesion size was 8.15 mm<sup>2</sup> in patients harboring the CC polymorphism and 7.05 mm<sup>2</sup> in patients with the CT polymorphism. The CC polymorphism was associated with larger lesions [21].

There is a limited number of studies about the effect of genetic polymorphisms on the response to therapy. Initial studies focused on photodynamic therapy (PDT). Seitsonen et al. studied the effect of *CFH* Y402H polymorphism on the response to PDT and found the frequency of the TT, TC, and CC polymorphisms among the good responders was 69.2%, 68%, and 58.3%, respectively [22]. Though the modality of treatment is different from our study, these results resemble ours: The TT polymorphism in our study was significantly higher among the good responders. We observed significantly more CC genotypes in the bad response group. There are conflicting results for ranibizumab therapy in the literature. Teper et al. found no significant effect of the *CFH* polymorphism on the response to intravitreal ranibizumab therapy [23]. McKibbin et al. found better response with the CC polymorphism [24]. Unlike our study, the CC allele was associated with a higher increase in VA, and patients harboring the CC polymorphism showed a poor response to bevacizumab therapy, as was the case in the present study. The CC genotype was associated with 10.5% and the TT and TC genotypes with 53.7% improvement in VA in Brantley et al.'s study as well [25]. Likewise, in our study the presence of the *CFH* Y402H CC allele was not associated with either an improvement in VA or better response to therapy. The association of the CC polymorphism with poor response to ranibizumab therapy was also documented in other studies [26-28].

Abedi et al. pointed out the association of *HTRA1* and *ARMS2* with a poorer visual outcome after anti-VEGF treatment in AMD [29]. *ARMS2* and *HTRA1* lie in the AMD

susceptibility locus identified on chromosome 10q26 and are expressed in the retina [29]. Genetic variation at this locus has been shown to confer a differential risk for CNV versus geographic atrophy. We have not had the chance to study these polymorphisms in our patient population, which is a limitation of our study.

In our study population, we identified a significant association between the *CFH* Y402H genotype and response to treatment for wet AMD with ranibizumab. The *CFH* Y402H CC genotype was associated with a poor response, whereas the TT genotype was associated with a good response to therapy in Turkish patients. Further research for this potential pharmacogenetic effect is necessary.

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