·Clinical Research·

Influence of CFH, HTRA1 and ARMS2 polymorphisms in the response to intravitreal ranibizumab treatment for wet age-related macular degeneration in a Spanish population

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Abstract

• AIM: To determine whether gene polymorphisms of the major genetic risk loci for age -related macular degeneration (AMD): ARMS2 (rs10490923), the complement factor H (CFH) (rs1410996) and HTRA1 (rs11200638) influence the response to a treatment regimen with ranibizumab for exudative AMD.

• METHODS: This study included 100 patients (100 eyes) with exudative AMD. Patients underwent a treatment with ranibizumab injections monthly during three months. Reinjections were made when the best corrected visual acuity (BCVA) decrease five letters (ETDRS) or central subfield retinal thickness gained 100 μm in optical coherence tomography image. Genotypes (rs10490923, rs1410996 and rs11200638) were analyzed using TaqMan probes or polymerase chain reaction-restricted fragment length polymorphisms analysis.

• RESULTS: There were no statistically significant differences in allelic distribution of CFH (rs1410996), ARMS2 (rs10490923) and HTRA1 (rs11200638) polymorphisms regarding to response to ranibizumab treatment.

• CONCLUSION: Ranibizumab treatment response is not related to CFH (rs1410996), ARMS2 (rs10490923) and HTRA1 (rs11200638) poymorphisms.

• **KEYWORDS:** age-related macular degeneration; polymorphisms; ARMS2; HTRA1; complement factor H; ranibizumab

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INTRODUCTION

Age-related macular degeneration (AMD) is a complex multifactorial disease. Genetic influence in the development of the disease have been demonstrated in several studies. First degree relatives of patients with AMD are at a higher risk of suffering the disease [1]. Heritability estimates for AMD is ranged from 46% to 71%. In the last decade multiple studies have analyzed the relationship between AMD and different gene polymorphisms. However, as different factors (for example, smoking, ultraviolet radiation, diet and so on) are associated to the onset of the disease, genetic variations only explain a small fraction of AMD susceptibility[2-3].

The most widely replicated loci are complement factor H (CFH) and the age-related maculopathy susceptibility-2 (ARMS2)/HtrA serine peptidase 1 (HTRA1) complex [4]. Multiple polymorphisms within the chromosome 10q26 region have been studied and their relationship with AMD susceptibility has been demonstrated in different gene variants such as ARMS2 A69S, the HTRA1 promoter variant rs11200638 adjacent to ARMS2 and a complex insertion/deletion variant in the untranslated region (UTR) of ARMS2 [4-5]. Different studies point that single nucleotide polymorphisms (SNPs) rs10490923 and rs11200638 have an influence in the risk of appereance of AMD [6]. Knowledge about the genetic influence in the apparition of AMD is continuously expanding. Multiple genetic variants have been studied in relation with AMD susceptibility, rare genetic variants in C3 and C9 genes as well as different variants in CFI gene have been associated with the disease[7].

AMD is the first cause of legal blindness in western population [3]. Vascular endothelial growth factor (VEGF) inhibition *via* injection of anti-VEGF monoclonal antibodies

(*e.g.* bevacizumab and ranibizumab) has become the gold standard for AMD treatment in the last decade based on findings from different studies [8-9]. In the last years new antiangiogenic treatments with different targets have appeared such as aflibercept, which is a fusion protein, combining the key binding domains of VEGF receptors 1 and 2 and the Fc portion of IgG[10].

Different treatment regimens have been investigated in order to minimize potential adverse effects and optimize the results of the treatment. Different studies found evidence to support the benefits of injections given every month for the first three months[8-9], but the treatment regimen following this period is a matter of debate. The Study of Ranibizumab in Patients With Subfoveal Choroidal Neovascularization Secondary to Age-Related Macular Degeneration (SUSTAIN) was one of the most important multi-centered clinical trials which intended to determine if injections "on demand" of patients response to the treatment could provide similar results to previous studies[10-11].

Because AMD has a strong genetic background. The knowledge of the influence of genetic variants in the treatment response may lead to a more individualized treatment based on pharmacogenomics. The purpose of the present study was to determine whether gene polymorphisms affect the response to a variable-dosing regimen treatment with ranibizumab (Lucentis, Genentech/Novartis, USA) in patients with choroidal neovascularization (CNV) subsequent to AMD. Gene variants selected for the study were ARMS2 (rs10490923), CFH (rs1410996) and HTRA1 (rs11200638). The influence of these polymorphisms on treatment efficacy was previously reported in patients undergoing photodynamic therapy (PDT) and bevacizumab treatment [11-15], and the effects of treatment with ranibizumab and CFH Y402H have been already studied[16]. We chose variants with demonstrated contribution to the disease in our population but not studied yet in relation with the treatment response[17].

SUBJECTS AND METHODS

A cohort of 100 consecutive patients (100 eyes; 58 women and 42 men; Caucasians; mean age, 76.62±8.4y) with the diagnosis of wet AMD at the Ophthalmology Service at the Hospital Clínico in Salamanca were included in the study. All patients underwent a complete eye examination, including best corrected visual acuity (BCVA) with Early Treatment Diabetic Retinopathy Study (ETDRS) charts, retinography, fluorescein angiography (FA), macular retinal thickness analysis measured by optical coherence tomography (OCT), slit-lamp examination, indirect ophthalmoscopy, and Goldmann applanation tonometry. Visual acuity was measured at 4 m by one tester after standardized refraction. OCT Stratus III, software version 4.0.2 (Zeiss Meditec, Dublin, CA, USA) was used to assess the retinal morphology (Retinal Thickness Map protocol) and central subfield retinal thickness (CSRT) (Fast Retinal Thickness Map protocol). All scans were acquired by the same doctor. FA and fundus photography (Visucam FF450+; Zeiss) were performed and interpreted by the same physician. Initial neovascular activity, and size and type of lesion (predominantly classic, minimally classic, occult) were assessed. Active subfoveal neovascularization was confirmed with FA and OCT at baseline. All patients had intraretinal cysts or subretinal fluid or both in the fovea. None of the patients were previously treated for AMD.

The study was a one-center, prospective cohort study. All patients were informed and signed a written informed consent before any study procedure was initiated. The study was approved by the Ethics Committee of the Hospital Clinico, Salamanca, Spain, and adhered to the tenets of the Declaration of Helsinki. We took peripheral blood samples to all the patients with wet AMD on the visit where they were diagnosed.

In our study, patients with subfoveal CNV subsequent to AMD underwent ranibizumab treatment with at least three injections. The SUSTAIN study [10] criteria were used to determine the need for reinjection after the first three monthly injections.

Visits were scheduled every month and subjects were reinjected when one of the following criteria was met: loss of 5 letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) charts compared to the highest number of letters during the first 3mo of the study; gain of more than 100 μm in CSRT compared to the lowest CSRT during the first 3mo of the study. A dose of 0.5 mg/0.05 mL ranibizumab was used for each treatment. This retreatment regimen was chosen for our study to unify the criteria of the different ophthalmologists in our department.

There were three treatment response criteria in all patients: 1) BCVA improvement: the patients gained at least 5 letters compared to BCVA at baseline; 2) OCT improvement: improvement of more than 100 μm in CSRT compared to the baseline measured by OCT.

All the patients underwent at least one and a half year follow up from the diagnosis (mean: 20.04±2.47mo). All the patients were treated with at least three initial injections except 1 patient had to abandon the treatment due to personal issues.

DNA collection, isolation, amplification and sequencing

DNA was isolated from peripheral blood following proteinase K, phenol-chloroform standard protocols. Allelic discrimination of the intronic polymorphism CFH c.2237-543 G>A (rs1410996) and the exonic polymorphism ARMS2 (LOC387715) c.8G>A/p.3R>H (rs10490923) were performed with TaqMan probes as provided by manufacturer with assays C_2530294_10 and C_29808405_20 respectively (Applied Biosystems, USA). Amplification conditions were

Table 1 Comparision of baseline demographic and clinical data of patients between treatment response groups $\bar{x} \pm s$

Variables	All patients	OCT improvement		*P*	BCVA improvement		*P*
		Yes	No		Yes	No	
Age (a)	76.62±8.4	74.47±6.5	75.86±7.2	0.754	77.23±4.7	74.21±8.1	0.342
Gender (M/F)	42/58	26/34	16/24	0.145	16/24	26/34	0.754
No. of injections	7.92±0.70	7.12±1.17	8.13±1.27	0.135	8.02±0.87	7.95±1.02	0.643
BCVA (ETDRS)	0.19±0.07	0.17±0.12	0.20±0.11	0.235	0.20±0.10	0.20±0.14	0.542
Follow-up (mo)	20.04±2.47	19.45±2.98	20.21±2.12	0.344	20.04±1.48	20.12±1.12	0.785
CSRT (μm)	332.62±16.59	344.46±17.88	329.45±18.33	0.154	334.35±19.36	327.41±15.89	0.576

BCVA: Best corrected visual acuity; CSRT: Central subfield retinal thickness. *P*: Yes *vs* No.

Table 2 Correlation between CFH (rs1410996) polymorphism distribution and treatment response

CFH (rs1410996)		Total	AA/AG/GG	*P*	AA+AG/GG	*P*	GG+AG/AA	*P*
BCVA improvement	Yes	40	2/24/14	0.324	26/14	0.133	38/2	0.768
	No	60	2/24/34		26/34		58/2	
OCT improvement	Yes	60	4/34/22	0.105	38/22	0.049	56/4	0.239
	No	40	0/14/26		14/26		40/0	

Table 3 Correlation between ARMS2 (rs10490923) polymorphism distribution and treatment response

ARMS2 (rs10490923)		Total	GG/GT/TT	*P*	GG+GT/TT	*P*	TT+GT/GG	*P*
BCVA improvement	Yes	40	10/20/10	0.885	30/10	0.676	30/10	0.700
	No	60	18/30/12		48/12		42/18	
OCT improvement	Yes	60	18/28/14	0.846	46/14	0.780	42/18	0.700
	No	40	10/22/8		32/8		30/10	

as follows: 95℃ initial denaturation for 3min followed by 35 cycles of 94℃ denaturation for 10s, 50℃ for CFH or 58℃ for ARMS2, annealing for 20s, and elongation at 72℃ for 40s. In all polymerase chain reaction (PCR) reactions a final elongation step was applied at 72℃ for 7min. Allelic discrimination of the promoter polymorphism HTRA1 625G>A (rs11200638) was performed by PCR-restricted fragment length polymorphisms (RFLP) after digestion of the PCR product with EagI restriction enzyme.

Statistical Analysis The Hardy-Weinberg equilibrium was used to determine that genotypes fell within a standard distribution. Baseline differences between genotype groups were tested (*i.e.* BCVA, OCT, number of injections) using the Kruskal-Wallis test. Analysis between two groups was made using Student's *t*-test, and ANOVA test used for more than two groups. Qualitative variables were analyzed with Pearson's χ^2 test. Magnitude of the association was expressed by the odds ratio (OR) and 95% confidence interval (CI). Statistics were performed with SPSS (version 19.0. SPSS Inc., Chicago, IL, USA). For statistical analyses, $P<0.05$ was considered to be statistically significant, although a Bonferroni correction for multiple testing was applied to SNP analysis, and $P<0.05/3$ (3: number of SNPs analyzed) were considered significant.

RESULTS

The differences in demographic (sex, age) and clinical data (BCVA preinjection, CSRT preinjection, number of injections and months of follow-up) between all the patients and the patients that OCT/BCVA improve or those who did not improve (Table 1) were not significant .

Analysis of the distribution of the intronic polymorphism c. 2237-543 G>A of the CFH gene (rs1410996) in relation to the response to ranibizumab treatment was studied following the improvement criteria previously described. There were not differences in genotype distribution according to OCT improvement, BCVA improvement or BCVA preservation (Table 2). When grouping patients expressing A-allele and comparing them to those homozygous for G (GG), we found that the patients that expressed A presented an increased probability of subjective (OR: 4.254) and OCT improvement (OR: 2.134)(Table 2). There were no such differences when analyzing BCVA.

The comparison of the distribution of genotypes and alleles of the c.8G>T polymorphism in the ARMS2 gene (rs10490923) demonstrated that there were no significant differences between the patients that response well to the treatment and those who did not well respond (Table 3).

The analysis of HTRA1 625G>A polymorphism (rs11200638) show no statistically significant difference in the genotype distribution between patients in relation with improvement criteria (Table 4).

A logistic regression adjusting by preinjection macular thickness measured by OCT and total number of injections was performed in order to find different confounding factors that could lead to inaccurate results and result that neither initial OCT or initial BCVA were confusion factors for our

Table 4 Correlation between HTRA1 (rs11200638) polymorphism distribution and treatment response

HTRA1 (rs11200638)		Total	GG/GA/AA	*P*	GG+GA/AA	*P*	AA+GA/GG	*P*
BCVA improvement	Yes	40	12/20/8	0.484	32/8	0.764	28/12	0.857
	No	60	22/20/18		42/18		38/22	
OCT improvement	Yes	60	20/26/14	0.809	46/14	0.658	40/20	0.734
	No	40	14/14/12		28/12		26/14	

study. This result, however, was non-significant when multiple correction testing was applied for each SNP analyzed (P=0.179).

DISCUSSION

CFH is an important regulator of the complement system and it has been showed that CFH and C3b/iC3b are located along with drusen, suggesting that these regions represent complement activating surfaces with drusen and Bruch's membrane [18]. Previous studies with CFH SNP's had demonstrated relationship between some CFH SNPs' and exudative AMD onset [19-22], therefore the response to intravitreal ranibizumab treatment could be related to CFH polymorphism. When the distribution of CFH (rs1410996) polymorphism was not statistically significant differences in genotypic distribution with BCVA or OCT improvement, although A allele carriers tended to a better response to the treatment. GG genotype, which had been demonstrated to increase the disease risk in Spanish population is, in this case, a worse response indicator [17]. There are similar results with different CFH polymorphisms regarding to BCVA, although there were no studies measuring OCT improvement and not another studies regarding to this polymorphism and ranibizumab response.

Inflammatory complement cascade is thought to favor the apparition of exudative AMD and associated neovascularization and inflammation [23]. The fact that this polymorphism is located in intron 14 and does not modify the protein sequence suggests that this polymorphism could regulate the expression of CFH gene or other nearby gene that could be related to an improved treatment response. A previous study did not find relationship between ranibizumab response and CFH Y402H [24-25]. Despite the polymorphism in this study is intronic, there were not statistically significant differences in the genotype distribution, confirming previous studies where no relation between CFH polymorphisms and response to the treatment were found[26].

There were not statistically significant differences in the relationship between ARMS2 (rs10490923) polymorphism and response to ranibizumab treatment. In the studies reported to the date ARMS2 polymorphisms are not associated to treatment response; however, worse responses were observed in those patients with haplotypes which included risk polymorphisms of CFH and ARMS2 [17,27-28]. Ranibizumab produces inhibition of inflammation and VEGF expression, meanwhile, the increase of AMD risk produced by ARMS2 alterations acts in a different way, which could explain that our results are not significant. A recent study showed rs1040924 polymorphism to be an important predictor of improvement in visual accuity after ranibizumab treatment [26]. This study drawed opposite results with a different polymorphism (rs10490923), although we did not find significance, the patients expressing the "risk genotype" (GG) tended to a better response to the intravitreal treatment in both OCT and BCVA improvement and in BCVA preservation.

We did not find differences in the response to ranibizumab in relation with HTRA1 (rs11200638) polymorphism. There are no studies related to this polymorphism and ranibizumab response to the date. Analysis of HTRA1 625G>A polymorphism (rs11200638) that is located on HTRA1 promoter region and has been associated to an increased expression[29], did not show any association with the response to antiangiogenic treatment. We did not find differences in the distribution of the polymorphism between positive and negative responders although we observed that carrying the risk genotype AA was more frequent among the negative responders.

The main limitation of our study is the number of patients, although 100 subjects is a significant number for a one-center study in Spanish population. The other limitation is the difficulty of performing statistical analysis in genotype distribution. The possibility of a type I error remains an important concern in genetic studies and performing many statistical tests may lead to a false positive finding. However, the best approach to this problem has been controversial[30]. In our case, the fact that these genes were associated with AMD risk in our population make the analysis of them and treatment response relevant in our population and decreases the possibility of this kind of error.

Previous studies have focused the measurement of the treatment response in BCVA [25] and found that initial BCVA was a confusion factor for the statistical analysis, in our case, we found that initial macular thickness in OCT, initial BCVA or total number of injections were not confusion factors for our results. Our study focused in other measurement parameters besides visual acuity, in order to find an explanation to the different response to the treatment and different genotypes that were pointed but not confirmed in previous investigations.

The genetic contribution to the variable outcomes in wet

AMD treatment is probably related to many loci. The combined effects of different variants and gene-environment interactions make it difficult to detect stronger associations. Single genotypes are likely to explain only a small proportion of the different response to the treatment. AMD has a complicated etiology, and therefore, different risk factors such as lifestyle or tobacco should be included in future studies about genetics and AMD treatment response.

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