Original article

Influence of CFH, HTRA1 and ARMS2 haplotype polymorphisms in the development of age-related macular disease

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**ABSTRACT**

Objective: To demonstrate genetic influence on the onset of age-related macular disease (AMD), analyzing genotype distribution of haplotypes, including polymorphisms of genes with proved relationships with AMD risk (CFH, ARMS2, HTRA1) in patients with AMD and in healthy people.

Methods: We took 101 consecutive patients with an AMD diagnosis following Wisconsin international classification. For our control group, we took 91 patients without AMD or any significant macular changes. We analyzed CFH rs 1410996, ARMS2 rs 10940923 polymorphisms using real time PCR with Taqman probes, and HTRA1-625 using restriction endonuclease digestion.

We studied haplotypes by simultaneously combining genotypes which, in previous studies, had been shown to have relationship with AMD (CFH, ARMS2, HTRA1) in patients with AMD and healthy people.

Results: There was a statistically significant higher proportion of patients with AMD simultaneously expressing CFH GG (rs 1410996) and ARMS2 TT (rs 10940923) (p = .037; OR: 7.742 [1.010–63.156]); ARMS2 TT (rs 10940923) and HTRA1-625 TT (p = .001; OR: 9.006 [2.019–40.168]) and CFH GG (rs 1410996), ARMS2 TT (rs 1040923) and HTRA1-625 GG (p = .043; OR: 6.702 [1.003–55.565]) genotypes.

Conclusions: Haplotypes which combine “risk genotypes”, demonstrated in previous studies, of our analyzed polymorphisms are more frequent in patients with AMD than in the control group, and they seem to increase the risk of suffering the disease in our population.

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Influencia de haplotipos de polimorfismos de CFH, HTRA1 y ARMS2 en la aparición de degeneración macular asociada a la edad

RESUMEN

Propósito: Demostrar la influencia genética en el desarrollo de degeneración macular asociada a la edad (DMAE) analizando las distribuciones genotípicas de haplotipos de polimorfismos de genes con relación demostrada con la aparición de DMAE (CFH, ARMS2, HTRA1) en pacientes con DMAE y personas sanas.

Método: Se tomaron 101 pacientes diagnosticados de DMAE (74 exudativa y 27 atrófica) según las normas del sistema internacional de clasificación Wisconsin. Como control se tomaron 91 pacientes sin DMAE ni otras alteraciones maculares. Se analizó el polimorfismo rs 1410996 del gen CFH, el rs 10940923 de ARMS2 mediante PCR a tiempo real con sondas Taqman y el HTRA1-625 mediante digestión con endonucleasas de restricción.

Se estudió la presencia de haplotipos que combinaban los genotipos que habían demostrado aumentar el riesgo de DMAE de los polimorfismos estudiados de CFH, HTRA1 y ARMS2 en estudios previos en nuestro grupo de pacientes y el grupo control.

Resultados: Se demostró que es más frecuente en el grupo de pacientes, de forma estadísticamente significativa, la expresión simultánea de los genotipos GG de CFH (rs 1410996) y TT de ARMS2 (rs 10940923) (p = 0,037; OR: 7,742 [1,010-63,156]); TT de ARMS2 (rs 10940923) y GG de HTRA1-625 (p = 0,001; OR: 9,096 [2,019-40,168]) y GG de CFH (rs 1410996), TT de ARMS2 (rs 10940923) y GG de HTRA1-625 (p = 0,043; OR: 6,702 [1,003-55,565]).

Conclusiones: La presencia de haplotipos que combinan genotipos, considerados de riesgo en estudios previos, de los polimorfismos analizados es más frecuente en pacientes con DMAE y parece aumentar el riesgo de padecer la enfermedad en nuestra población.

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Introduction

Age-related macular disease (AMD) is a disorder of the photoreceptor–retina pigment epithelium–Bruch membrane–choriocapillaries complex.\(^1\)–\(^3\)

The importance of AMD lies in its prevalence and incidence as it constitutes the main cause of legal blindness in adults of European origin.\(^4\)–\(^7\) In addition, AMD is the third cause of blindness throughout the world, accounting for 8.7% of all currently blind individuals.\(^8\) In the Western world it is the main cause of irreversible blindness in the age group comprised between 65 and 74 and the second cause in the group between 45 and 69 years of age, although the numbers vary according to the area of the world where studies were carried out. In 2000 it was estimated that, for 54% of all legally blind Caucasian people in the United States over 40 years of age, the cause was AMD followed at a distance by cataracts with 9%. It must be taken into account that these numbers are not applicable to the Afro-American population in which AMD is the third cause of blindness.\(^9\) In Australia it was estimated that the cause of blindness for 13% of legally blind pensioned people was AMD.\(^10\)

Genetic influence in the pathogenic of AMD is determined by means of studies in families and twins.\(^11\)–\(^18\) Compared with first-degree relatives in families who do not have the disease, first-degree relatives of AMD patients are at greater risk of developing the disease\(^17\) in addition to being affected at an earlier age\(^16,19\) and having greater probabilities of developing advanced AMD.\(^16\)

In order to determine the relative contribution of inheritance and the environment to the etiology of AMD, Seddon et al. carried out a study based on AMD population in twins, including matching and non-matching as well as monozygot and dizygotic.\(^20\) Estimates about the inheritability of AMD gave statistically significant results, with a range of between 46% and 71%. These results justified the need to begin a search of genes related to AMD despite the initial difficulties seemingly involved in the genetic analysis of such a complex disease with late expression.

The progress made in the past decade in the study of macular and retinal dystrophy with monogenic inheritance has provided significant pointers to begin a study of AMD genetics. The similarities between the phenotypic expression of hereditary disease which appear early in life with some of the late forms of disease is similar to AMD suggested a potential relationship of candidate genes with AMD. In addition, the said genes were selected on the basis of results of association studies (position criteria) and knowledge on the function of genes (functional criteria). However, this approach has not produced significant developments.\(^21\) Evidence of direct association with the disease has been found in some of the genes.\(^21\) Should these results be confirmed, the variations of said genes would be related to only a small fraction of the vulnerability to the disease.

Complement H factor gene

Multiple analyses of complete genome linking and the meta-analysis of Fisher et al. pointed towards the presence of a
locus in 1q25-q31 associated to the disease.22-27 Recent studies of cases and controls have identified complement factor H (CFH) as the causing gene.28-30 It has been consistently demonstrated that variant CFHY402H, located in a fixation site for reactive protein C (RPC), exhibits a significant association with the appearance of AMD.31-33

CFH is an important regulator of the complement cascade. There are 3 enzymatic cascades: the classical complement pathway initiated by antigen–antibody complexes and surface RPC, the lectin pathway and the alternative complement pathway which is activated by surface C3b. All these pathways converge at the point in which C3 is divided into C3A and C3B by C3 convertase, which activates C5 convertase which leads to the formation of the membrane attack complex by means of terminal components (C5b-C9). CFH specifically inhibits the attentive cascade of the complement. It links to C3b and acts as a cofactor in proteolysis of C3b through factor I, which prevents the formation of C3 convertase in the alternative pathway as well as the production of C5 convertase in the common complement pathway. As a result, CFH interferes with the progress of the entire cascade.34-37 Hageman et al. demonstrated that CFH and C3b/IC3b are located between the drusen, suggesting that these regions represent complement cascade activation surfaces between drusen and Bruch membrane.38,39

In a prospective study based on a population designed with 5681 subjects, researchers of the Rotterdam Eye Study demonstrated that CFH is associated to all AMD phases, from the first signs of early AMD to drusen up to the late lesions that cause significant vision loss.38 The risk increases in each successive phase of AMD.

A recent study suggests that there may be multiple susceptible alleles in the chromosome region of the CFH gene with unencoded variants playing an important role in the susceptibility to the disease.39 In a group of 544 affected patients without familial relationship and 268 healthy controls, Li et al. analyzed the impact of 84 polymorphisms on the susceptibility to the disease located in a region of 123 kb which overlaps gene CFH, finding a strong association between the state of the disease and Y402H polymorphism (rs 1061170). Twenty additional variants exhibited an even stronger association. Accordingly, the authors suggest that many haplotypes in this region could regulate the risk of developing AMD. Due to the fact that the polymorphisms which exhibited the strongest association with the susceptibility to AMD did not seem to affect the primary sequence of the CFH protein, the authors speculated with the possibility that these variants could be important in the regulation of CFH expression, of other nearby complement genes, or both.39

Susceptibility gene for age-related maculopathy type 2 (ARMS2/LOC387715)

Two recent studies have identified locus LOC387715/HTRA1 in 10q26 as the second most important locus in the pathogenicity of AMD.40,41 The study by Rivera found the strongest association of LOC387715 with an increased AMD risk, demonstrating an increased probability of developing the disease of 7.6 in homozygote individuals for the risk allele. These findings were confirmed in an independent case and control study. In addition, the said study demonstrated once again the strong association between AMD and the CFH variant that encodes Y402H. The results indicated an independent contribution vis-à-vis the general risk of the disease of the effects of risk alleles in LOC387715 (Ala69Ser) and the CFH gene locus (Tyr402His). Recently, these findings were independently confirmed by various authors.42-45

No differences were found in the distribution of risk alleles in LOC387715 between high risk early onset AMD and advanced AMD patients. This was also demonstrated for geographic atrophy and the exudative form of the disease. At present it is not known whether the LOC387715 and CFH risk alleles have a direct correlation with the severity of the disease or with some clinical expression thereof that could be subject to therapeutic intervention.46

The functional implications of ARMS2 polymorphism rs 10490924 and its association with AMD were studied by means of cell construction analysis and RT-PCR.47,48 Even though the specific function of ARMS2 is not known, it was demonstrated that the protein was located in the external mitochondrial membrane.47,48 It was also demonstrated that the stability, expression and location of the proteins was not affected by this variant in mammals. The hypotheses arose that variant rs 10490924 of gene ARMS2 could affect the conformation of the proteins and modify mitochondria functions.47

A recent study based on cell culture indicated that ARM2S is distributed in the cytosol and not in the external mitochondrial membrane.49 This would imply that the risk attributed to ARMS2 could include other non-mitochondrial pathways.

High temperature requirement serin peptidase 1 (HTRA1)

Studies carried out in parallel to those made in regions near the ARMS2 region singled out HTRA1 as one of the genes related to susceptibility to AMD in chromosome 10q26. Based on the location of ARMS2 polymorphism rs 10490924 between PLEKH1 and HTRA1, and the fact that ARMS2 has a low homology sequence between species, it was suspected that this simple nucleotide polymorphism (SNP) could be related to a variant of a different gene.50 Repeating the sequencing of PLAKHA1 and HTRA1 in a case and control study, an SNP was identified in HTRA which had a significant relationship with rs 10490924.

The involvement of HTRA1 was subsequently confirmed in a Chinese cohort51 which immunologically marked HTRA1 antibodies. These gave intense staining in drusen of AMD patients. In addition, a greater expression of HTRA1 mRNA (messenger RNA) was observed in the risk allele of rs 1200638 in lymphocytes and the retina pigment epithelium of patients with the disease.51 The risk of disease associated to this gene was demonstrated to be greater among Asians52-55 when compared to Caucasians.51,56 A meta-analysis of the HTRA1 promoter based on polymorphisms of 14 case and control studies indicated a strong association with AMD, with high risk in homozygote subjects for the risk allele.57
Patients and methods

For the study patients having any type of AMD were selected according to the criteria of the Wisconsin classification system. After obtaining an informed consent according to the legal regulations for clinical studies in Spain and to the Ethics Committee rules of the Salamanca University Hospital, peripheral blood samples were taken of all the patients diagnosed with AMD by the Ophthalmology Service of the Salamanca University Clinic Hospital between September 1, 2007 and September 4, 2009.

Overall, 101 patients were recruited for the study (56 males and 45 females) (p < 0.01) with a mean age of 77.5 years (±7.198). Of all the patients, 74 were diagnosed with wet AMD while 27 exhibited the atrophic form. Of the former, 50 were treated with intravitreal ranibizumab injection.

All the patients underwent a full assessment in the practice, including data capture comprising risk factors, visual acuity, anterior and posterior pole exploration, retinography in both eyes and optic coherence tomography (Retinograph: TRC-50DX Mydriatic Retinal Camwas, Topcon [Topcon Medical Systems, EE.UU], OCT: Stratus OCT, Zeiss [Zeiss Technologies, EE.UU]). Fluorescein angiography was carried out when active neovascularization was suspected.

The control group comprised 91 subjects, 52 females and 39 males, with a mean age of 70.51 years (±6.386). Sex had no significant difference in our patients (p > 0.05) who were assessed in the Ophthalmology Service of the Salamanca Clinical Hospital and were found to be free of AMD or significant macular alterations.

A single nucleotide polymorphism (SNP) study was made for each of the genes (CFH, ARMS2, HTRA1) by means of real-time RPC utilizing fluorochrome-marked probes. In the RPC with Taqman probes, the amplification and detection processes occurred simultaneously in the same closed tube without requiring subsequent action. In addition, utilizing fluorescein detection it was possible to measure during amplification the amount of formed DNA. This enabled us to identify and record at all times the kinetics of the amplification reaction. The thermocyclers utilized to carry out the RPC with Taqman probes comprise a fluorescence meter and are designed to measure at any point in time the fluorescence issued in each of the tubes where the amplification is carried out. For allele discrimination, specific fluorochrome-marked probes were utilized.

Results

The approach was to determine the risk of developing AMD if the subject exhibited at the same time several genotypes of the study which have demonstrated to increase vulnerability to the disease (Table 1).

When grouping the subjects that simultaneously expressed genotype GG of the rs 1410996 polymorphism of gene CFH and genotype GG of the HTRA1-625 polymorphism, no significant differences were found in the distribution between patients and controls (Table 2), even though a tendency of the group of patients to express the haplotype more frequently was identified.

When grouping the subjects that expressed at the same time the “risk” genotypes of the rs 1410996 polymorphism of gene CFH (GG) and the rs 1040924 polymorphism of gene ARMS2 (TT), significant differences were found in the distribution between the group of control and patients. In the latter, haplotype GGTT was more frequent (Table 2).

When grouping the subjects who expressed genotype TT of the rs 1040924 polymorphism of ARMS2 and genotype GG of HTRA1-625, differences were found in the distribution between the control group and the patients group. The presence of the haplotype was more frequent in the group of patients (Table 2).

When grouping the subjects in which the GG genotypes of the rs 1410996 polymorphism of CFH, TT genotype of the rs 1040924 polymorphism of ARMS2 and GG genotypes of the HTRA1-625 polymorphism, significant differences were found in the distribution of the haplotype, which was present more frequently in the group of patients (Table 2).

Discussion

The first important finding in the genetic association and AMD studies was the discovery of a complement factor H (CFH) located in the complement regulation locus in chromosome 1q31.3.30,39,43,60,61 This gene encodes the CFH protein which is an important inhibitor of the complement cascade and acts both in the standard as well as the alternative pathway. Therefore, the absence of CFH or a reduced activity thereof can lead to the complement activation which acts as a stimulus for the formation of drusen.62 In addition, environmental risk factors are associated to AMD such as tobacco diminish blood CFH levels.62

Recent studies have demonstrated that a polymorphism located in exon 9 of the CFH gene (rs 1061170 or Y402H) is associated to the appearance of soft drusen as well as with an increased risk of exudative AMD.28,33,43,44,63

In order to understand the fundamental role of CFH variances in the disease studies were carried out analyzing the complete CFH GEN genome region and its adjacent regions to assess their contribution to the susceptibility of developing the disease.30,43 Multiple SNP were found both in the encoding as well as the non-encoding regions which contributed to increase the risk of the disease. The said SNP exhibited stronger associations than rs 1061170, indicating the potential role of other determining factors, particularly the non-encoding variants, in the vulnerability to AMD.39,43

Recently it has been published that an intronic SNP (rs 1410996) exhibits strong association with AMD.43 Considering that very few studies had been made about this polymorphism, it was decided to include it in our research. Accordingly, we can state that allele G in the rs 1410996 polymorphism increases the risk of AMD in our population. This result matches those of previous studies.43

A second AMD susceptibility gene was found in chromosome 10, position 10q26, LOC387715 (T allele in the rs 1040924 polymorphism).43,61 Its function is unknown, although it is believed to play an important role in mitochondrial function.
A gene which is very close to LOC387715, PLEKHA 1, is related to cell immunity. The relationship of this gene with AMD was verified in multiple studies.\(^{23,25,64,65}\)

It was demonstrated that the relationship with the appearance of the disease was highly significant, to the extent that LOC387715 became known as the AMD type II susceptibility gene (ARMS2).\(^{41,44}\) When demonstrating the relationship of ARMS2 with AMD it was verified that, in contrast with other loci related to the disease where only one gene is identified (CFH, C3),\(^{28,43,63}\) in this 10q26 region 3 genes were discovered having a consistent association with the appearance of the disease.\(^{33,40,44,66}\) Recent data have reduced the amount of genes related to AMD susceptibility in this chromosome region to 2, i.e., ARMS2 and HTRA1. It is believed that a diminished expression of ARMS2 would relate to increased risk of the disease. Our data match those of multiple studies that confirm that the expression of allele T increases the risk of developing the disease,\(^{40,44}\) as in the results obtained in the said studies are very similar to our own.

HTRA1 is the other gene in the 10q26 region which demonstrated involvement with the susceptibility of developing AMD.\(^{44,67,68}\) Initially, it was thought that an increase in the expression of HTRA1 due to some of its SNP enhanced the risk of developing AMD.\(^{44,67,68}\) But in-depth studies demonstrated that the increased risk of developing the disease associated with this region is regulated by multiple variants including an increased expression of HTRA1 and, as explained above, diminished expression of ARMS2. We studied one of the HTRA1 polymorphisms (HTRA1-625) which seems to be involved in the increased risk of developing AMD. Analyses of the effects of this polymorphism in the HTRA1-promoting activity in some of the studies demonstrated the existence of an increased expression\(^{46}\) which enhanced the appearance of the histological changes produced with the disease, while in others these differences were not found and no increase of HTRA1 messenger RNA expression was found in the retina of patients with AMD vis-à-vis controls.\(^{47}\) The results of this study match those obtained in others because an increase in the expression of the GG genotype involves an increase in the expression of HTRA1 which could be associated to increased susceptibility of developing AMD.\(^{52,53,55}\) These results confirm other studies carried out to date\(^{56,57}\) in which the expression of allele G is considered as a risk factor for the disease whereas the expression of allele A, mainly in the homozygote genotype, seems to diminish the probability of developing AMD.

It was considered whether the simultaneous existence of several genotypes that has been proven to significantly increase the risk of developing the disease would increase susceptibility to AMD. Grouping patients with the risk genotype for HTRA1-625 and rs 1410996 of CFH, no significant differences were found (Table 2). However, an increased risk of developing AMD was found when grouping patients

### Table 1 – Differences in genotype distribution of CFH polymorphisms HTRA1 and ARMS2 in patients with age-related macular degeneration and healthy controls.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Patients (n = 101)</th>
<th>Controls (n = 91)</th>
<th>(p) OR (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFH (rs 1410996)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>7 (6.9%)</td>
<td>15 (16.5%)</td>
<td>0.038</td>
</tr>
<tr>
<td>AG+GG</td>
<td>54 (53.5%)</td>
<td>65 (71.4%)</td>
<td>2.010 (1.030–6.381)</td>
</tr>
<tr>
<td>AA+AG</td>
<td>47 (46.5%)</td>
<td>26 (28.6%)</td>
<td>2.176 (1.194–3.964)</td>
</tr>
<tr>
<td>LOC387715/ARMS2 (rs 10490923)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>18 (17.8%)</td>
<td>3 (3.3%)</td>
<td>0.001</td>
</tr>
<tr>
<td>GT+GG</td>
<td>83 (82.2%)</td>
<td>88 (97.6%)</td>
<td>6.369 (1.801–22.234)</td>
</tr>
<tr>
<td>TT+GT</td>
<td>37 (36.6%)</td>
<td>60 (65.9%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GG</td>
<td>64 (63.4%)</td>
<td>31 (34.1%)</td>
<td>3.34 (1.848–6.060)</td>
</tr>
<tr>
<td>HTRA1 (rs 11200638)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>27 (26.7%)</td>
<td>9 (9.9%)</td>
<td>0.003</td>
</tr>
<tr>
<td>AG+AA</td>
<td>74 (73.3%)</td>
<td>82 (90.1%)</td>
<td>7.132 (4.467–11.238)</td>
</tr>
<tr>
<td>AA</td>
<td>24 (23.8%)</td>
<td>61 (67%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AG+GG</td>
<td>77 (76.2%)</td>
<td>30 (33%)</td>
<td>6.524 (3.463–12.28)</td>
</tr>
</tbody>
</table>

### Table 2 – Haplotype distribution of ARMS2, HTRA1 polymorphisms of CFH in patient and control groups.

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Patients (n = 101)</th>
<th>Controls (n = 91)</th>
<th>(p) OR (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGT (CFH/ARMS2)</td>
<td>8 (7.2%)</td>
<td>1 (1.1%)</td>
<td>0.037</td>
</tr>
<tr>
<td>Others</td>
<td>93 (92.8%)</td>
<td>90 (98.9%)</td>
<td>7.742 (1.010–63.156)</td>
</tr>
<tr>
<td>GGG (CFH/HTRA1)</td>
<td>12 (11.9%)</td>
<td>4 (4.4%)</td>
<td>0.071</td>
</tr>
<tr>
<td>Others</td>
<td>89 (88.1%)</td>
<td>87 (95.6%)</td>
<td>0.001</td>
</tr>
<tr>
<td>TT (ARMS2/HTRA1)</td>
<td>17 (16.8%)</td>
<td>2 (2.2%)</td>
<td>9.006 (2.019–40.168)</td>
</tr>
<tr>
<td>Others</td>
<td>84 (83.2%)</td>
<td>89 (97.8%)</td>
<td>0.043</td>
</tr>
<tr>
<td>GGT (CFH/ARMS2/HTRA1)</td>
<td>7 (6.9%)</td>
<td>1 (1.1%)</td>
<td>6.702 (1.003–55.565)</td>
</tr>
<tr>
<td>Others</td>
<td>94 (93.1%)</td>
<td>90 (98.9%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>
that simultaneously had the GG genotype of the CFH polymorphism rs 1410996 and the GG genotypes of ARMS2 polymorphism rs 1040924 (Table 2; OR: 7.742). In addition, increased susceptibility to the disease was found when grouping homozygotes for allele G of HTRA1-625 polymorphism and homozygotes for allele T of the ARMS2 rs 1040924 polymorphism (Table 2; OR: 9.006). When grouping the 3 “risk” genotypes, a greater risk of developing the disease was found with this haplotype (Table 2; OR: 6.702).

It is reasonable to think that the simultaneous presentation of 2 or 3 “risk” genotypes would increase the risk of developing the disease, although the results must be interpreted with caution due to the low number of subjects that present the various haplotypes, both in the group of patients and in the control group.

Accordingly, it can be concluded that the presence of haplotypes that combine genotypes of the analyzed polymorphisms considered to be of risk in previous studies is more frequent in patients with AMD and appears to increase the risk of developing the disease in our population.

Conflict of interest

No conflict of interest has been declared by the authors.

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