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Alleles in the HtrA Serine Peptidase 1 Gene Alter the Risk of Neovascular Age-Related Macular Degeneration

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Abstract

Objective—To examine if the genes encoding the pleckstrin homology domain-containing protein gene (PLEKHA1), hypothetical *LOC387715/ARMS2* gene, and HtrA serine peptidase 1 gene (*HTRA1*) located on the long arm of chromosome 10 (10q26 region) confer risk for neovascular age-related macular degeneration (AMD) in an independent or interactive manner when controlling for complement factor H gene (*CFH*) genotype and smoking exposure.

Design—Retrospective matched-pair case–control study.

Participants—Hospital clinic-based sample of 134 unrelated patients with neovascular AMD who have a sibling with normal maculae (268 subjects).

Methods—Disease status was ascertained by at least 2 investigators by review of fundus photographs and/or fluorescein angiography according to the Age-Related Eye Disease Study grading scale. If necessary, a home retinal examination was performed (n = 6). A combination of direct sequencing and analysis of 8 highly polymorphic microsatellite markers was used to genotype 33 megabases of the 10q26 region on leukocyte DNA. Smoking history was obtained via a standardized questionnaire and measured in pack-years. The family-based association test, haplotype analysis, multiple conditional logistic regression, and linkage analysis were used to determine significant associations.

Main Outcome Measure—Neovascular AMD status.

Results—Of the 23 variants we identified in the 10q26 region, 6 were significant. Four of the 6 were novel and included 2 genotypes that reduced risk of AMD. Many single-nucleotide polymorphisms (SNPs), including the previously reported variants rs10490924 (hypothetical

LOC387715/ARMS2) and rs11200638 (*HTRA1*), defined 2 significant haplotypes associated with increased risk of neovascular AMD. The coding *HTRA1* SNP rs2293870, not part of the significant haplotypes containing rs10490924 and rs11200638, showed as strong an association with increased susceptibility to neovascular AMD. Linkage analysis supported our findings of SNP association ($P < 10^{-15}$). No significant interactions were found between any of the SNPs in the 10q26 and smoking or between these SNPs and *CFH* genotype.

Conclusions—Independent of *CFH* genotype or smoking history, an individual's risk of AMD could be increased or decreased, depending on their genotype or haplotype in the 10q26 region.

Neovascular age-related macular degeneration (AMD) is characterized by the growth of abnormal new blood vessels beneath the retina that can cause severe and rapid vision loss due to hemorrhage and exudation. It is this more advanced form that is responsible for the majority of debilitating vision loss due to AMD. In the United States, it is predicted that about 3 million individuals over the age of 50 will have advanced AMD in at least one eye by 2020.¹

Current diagnostic methods focus on the detection of neovascular AMD, because available treatments are directed against this advanced stage of the disease. Although the newest treatments offer some chance of visual improvement, they require invasive delivery methods, cannot prevent disease, and have limited ability to reverse vision loss. Assessments of an individual's risk of developing advanced AMD are based on ocular findings in those who already have the early stages. Methods have yet to be discovered that determine which individuals are at highest risk of vision loss due to AMD prior to the development of any signs of the disease. The identification of genetic variants that could be used as biomarkers would help to predict risk of the more advanced stages of AMD. Moreover, discovering precisely which genes and environmental factors contribute to the pathophysiology of AMD and elucidating their modes of interaction could provide new targets for therapeutic or behavioral intervention, thereby reducing or preventing the incidence of this disease.

Although the complement factor H gene (*CFH*) Y402H variant on 1q32 appears to be the genetic risk factor most consistently associated with AMD,^{2–6} the 10q26 region, where the pleckstrin homology domain–containing protein gene (*PLEKHA1*), hypothetical *LOC387715/ARMS2* gene, and HtrA serine peptidase 1 gene (*HTRA1*) reside adjacent to one another (Table 1 [available at <http://aojournal.org>]), appears to be the most strongly associated overall with AMD susceptibility.⁷ Although many reports have demonstrated that variant rs10490924 in hypothetical *LOC387715/ARMS2* gene is associated with all types of AMD,^{8–11} it was recently shown that the single-nucleotide polymorphism (SNP) rs11200638 in the *HTRA1* promoter, in linkage disequilibrium (LD) with rs10490924, is likely the causal variant.^{12–14} Moreover, data from the Age-Related Eye Disease Study showed a significant association between the SNP rs1045216 in *PLEKHA1* and increased risk of neovascular AMD.¹¹

In the study presented here, we genotyped 33 megabases of the 10q26 region (Table 1) in samples from unrelated patients with neovascular AMD who had one sibling with normal maculae and was past the age at which the affected sibling was diagnosed with neovascular AMD. Our phenotypically well-defined cohort of 268 subjects comprised 134 extremely

discordant sibpairs—that is, pairs with one member in the upper 10% of disease severity (affected sibling) and the other member in the bottom 10% to 30% of disease severity (unaffected sibling).^{15,16} We have previously demonstrated that such types of sibpairs can be powerful in identifying the contribution that genes and environmental factors make simultaneously to AMD susceptibility.¹⁷ We sought to validate previous findings and possibly identify novel variants in the 10q26 region using a combination of direct sequencing and analysis of highly polymorphic microsatellite markers tightly linked to the genes of interest. For each subject, we directly sequenced exon 12 of *PLEKHA1*, the entire putative coding region of *LOC387715/ARMS2*, and the promoter region and entire coding region of *HTRA1* to identify the contribution that allelic risk factors such as those reported in the 10q26 region make independently and in combination with the factors most consistently associated with AMD susceptibility, *CFH* Y402H genotype^{2–6,18} and smoking.¹⁹

Materials and Methods

Patient Population

The protocol was reviewed and approved by the institutional review boards at the Massachusetts Eye and Ear Infirmary, Boston, Massachusetts, and William Beaumont Hospital, Royal Oak, Michigan, and conforms to the tenets of the Declaration of Helsinki. Eligible patients were enrolled in this study after they gave informed consent in person, over the phone, or through the mail before answering a standardized questionnaire and donating 10 to 50 ml of venous blood. In most cases, the donation of venous blood from both patients and their siblings was made on the same day that fundus photographs and/or fluorescein angiographs were obtained.

Details of the recruitment of patients and their siblings are described elsewhere.²⁰ In brief, index patients with neovascular AMD were recruited from the Retina Service of the Massachusetts Eye and Ear Infirmary and Associated Retina Consultants at the Beaumont Hospital. All index patients were 50 years or older and had the neovascular form of AMD in at least one eye, defined by subretinal hemorrhage, fibrosis, or fluorescein angiographic presence of neovascularization documented at the time of or before enrollment in the study. Patients whose only exudative finding was a retinal pigment epithelium detachment were excluded because this finding may not represent definite neovascular AMD and, therefore, the severe phenotype we sought. Patients with signs of pathologic myopia, presumed ocular histoplasmosis syndrome, angioid streaks, choroidal rupture, any hereditary retinal diseases other than AMD, and previous laser treatment due to retinal conditions other than AMD were also excluded.

The unaffected siblings had normal maculae at an age older than that at which the index patient was first diagnosed with neovascular AMD. Normal maculae (defined as the zone centered at the foveola and extending 2 disc diameters [3000 μ m] in radius) fulfilled the criteria of 0 to 5 small drusen (all <63 μ m in diameter), no pigment abnormalities, no geographic atrophy, and no neovascularization (as defined previously^{21,22}; AMD level 1 on the Age-Related Eye Disease Study scale). Disease status of every participant was confirmed by at least 2 of the investigators (IKK, JWM, TPD) by evaluation of fundus photographs or fluorescein angiograms, except when one of the investigators directly

examined an unaffected sibling during a home visit (n = 6 cases). Subject characteristics of our extremely discordant sibpair population are presented in Table 2 (available at <http://aaojournal.org>).

Ascertainment of Smoking Exposure

We administered a standardized questionnaire to all eligible participants in person or over the phone to ascertain smoking exposure, with the age of the index patient at the time of the fundus photographs as our cutoff reference age for smoking exposure for all members in the sibpair. In most cases, the diagnosis of AMD was made simultaneously with the diagnosis of neovascular AMD. If a participant ever smoked, we recorded the age when they started smoking, age when they quit (if they did quit), and number of packs of cigarettes smoked per day, on average. Based on the responses, the number of pack-years of cigarettes smoked was calculated for each smoker. Participants who smoked <100 cigarettes during their lifetime (i.e., <1/73 of a pack-year) were categorized as having never smoked. A pack-year was defined as 1 pack of cigarettes per day for 1 year, with 1 pack defined as 20 cigarettes. For our statistical analysis (see below), the reference cutoff for smoking was defined as 10 pack-years, versus <10 pack-years. With this cutoff, our subjects were divided into 2 approximately equal groups.¹⁷

Genotyping Analysis

Leukocyte DNA was purified by using either the standard phenol-chloroform or the DNAzol (Invitrogen Corp., Carlsbad, CA) extraction protocol. Previously reported oligonucleotide primers were used to amplify the coding region and flanking intronic sequences of exon 12 for *PLEKHA1*,⁹ exon 9 of *CFH*,⁴ and the promoter sequence for *HTRA1*.¹² For the putative *LOC387715/ARMS2* gene region (including both exons) and the 9 exons of *HTRA1*, oligonucleotide primers were selected using the Primer3 program (<http://primer3.sourceforge.net>) to encompass the entire coding region and flanking intronic sequences (Table 3 [available at <http://aaojournal.org>]).

For all 4 genes, polymerase chain reaction (PCR) was used to amplify genomic DNA fragments from 20 ng of leukocyte DNA in a solution of × 10 PCR buffer containing 25 mmol/l of MgCl₂; 0.2 mmol/l each of deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate; and 0.5 U of Taq DNA polymerase (USB Corp., Cleveland, OH). For the *PLEKHA1* and *HTRA1* genes, 5 mol/l of (carboxymethyl) trimethylammonium inner salt was added to each PCR (Sigma-Aldrich, St. Louis, MO). The temperatures used during the PCR were as follows: for *PLEKHA1* and *HTRA1*, 95° C for 5 minutes, followed by 35 cycles of 60° C for 30 seconds, 72° C for 30 seconds, and 95° C for 30 seconds, with a final annealing at 60° C for 1.5 minutes and extension of 72° C for 5 minutes; for *LOC387715/ARMS2*, 95° C for 5 minutes, followed by 35 cycles of 62° C for 30 seconds, 72° C for 30 seconds, and 95° C for 30 seconds, with a final annealing at 62° C for 1.5 minutes and extension of 72° C for 5 minutes; for *CFH*, 95° C for 5 minutes, followed by 35 cycles of 56° C for 30 seconds, 72° C for 30 seconds, and 95° C for 30 seconds, with a final annealing at 56° C for 1.5 minutes and extension of 72° C for 5 minutes. For sequencing reactions, PCR products were digested according to the manufacturer's protocol with exonuclease I and shrimp alkaline

phosphatase (USB), then were subjected to a cycle sequencing reaction using the Big Dye Terminator 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Products were purified with Performa DTR Ultra 96-well plates (Edge Biosystems, Gaithersburg, MD) to remove excess dye terminators. Samples were sequenced on an ABI Prism 3100 DNA sequencer (Applied Biosystems). Electropherograms generated from the ABI Prism 3100 were analyzed using the Lasergene DNA and protein analysis software (DNASTAR, Inc., Madison, WI). Electropherograms were read by 2 independent evaluators without knowledge of the subject's disease status. All patients were sequenced in the forward direction (5'–3') unless variants, polymorphisms, or mutations were identified, in which case confirmation was obtained in some cases by sequencing in the reverse direction.

Genotyping of Microsatellite Markers

We analyzed 8 highly heterozygous microsatellite markers spanning 33 megabases of the 10q26 region (Table 1 [available at <http://aaojournal.orgx>]). These markers included several that were tightly linked to *PLEKHA1*, *LOC387715/ARMS2*, and *HTRA1* (Tables 3, 4 [available at <http://aaojournal.org>]). All markers were fluorescently labeled with either 5-carboxyfluorescein or 6-carboxyfluorescein on the 5' end of the reverse primer, and an additional sequence of CTGTCTT was added to the 5' of the forward primer. The PCR was used to amplify genomic DNA fragments from 20 ng of leukocyte DNA in a solution of $\times 10$ PCR buffer containing 25 mmol/l of $MgCl_2$; 0.2 mmol/l each of deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate; and 0.5 U of Taq DNA polymerase (USB). The temperatures used during the PCR were 95° C for 5 minutes, followed by 35 cycles of 54° to 60° C (specific to primer pair) for 30 seconds, 72° C for 30 seconds, and 95° C for 30 seconds, with a final annealing at 54° to 60° C (specific to primer pair) for 1.5 minutes and extension of 72° C for 5 minutes. Polymerase chain reaction products were diluted 1:20 for markers labeled with FAM and 1:10 for markers labeled with HEX. Samples were pooled according to product size and denatured before being genotyped on the ABI 3730xl DNA Analyzer (Applied Biosystems). Data were then analyzed using ABI's Genemapper 3.7 software for analysis, which interrogates the quality of the size standard and makes the appropriate genotype calls based on size. For quality control purposes, all genotypes were then evaluated manually.

Statistical Analyses

The program FBAT (<http://biosun1.harvard.edu/~fbat/fbat.htm>), which tests for family-based association, was used to evaluate the effect of each SNP individually on risk of AMD.²³ Single-nucleotide polymorphisms were included for analysis in FBAT only if the minor allele frequency in the affected and unaffected siblings combined was $\geq 5\%$ and the number of informative families was at least 4. A Bonferroni correction was applied to the pointwise *P* values that were calculated for each allele of the 14 SNPs that met these criteria.

Haploview (<http://www.broad.mit.edu/mpg/haploview>) was used to generate the LD plot (Fig 1 [available at <http://aaojournal.org>]) among the 19 identified SNPs that had a minor allele frequency $\geq 5\%$.²⁴ Linkage disequilibrium (r^2) between each of the 19 SNPs is depicted in Figure 1. The haplotype blocks were constructed by Haploview using the

method proposed by Gabriel et al.²⁵ Individual haplotypes were inferred and tested for association with AMD using FBAT.²³

Conditional logistic regression (CLR) (SAS 9.1 [<http://www.sas.com>]) was performed to identify factors associated with wet AMD. Potential risk factors of interest, as defined above, were evaluated initially one at a time. A multiple CLR model for each significant SNP in the 10q26 region was built using those factors from the single factor model that appeared to be associated with neovascular AMD with $P \leq 0.1$. The *CFH* Y402H CT genotype was kept in the models, although it has $P > 0.1$, to adjust the effect of *CFH* more precisely. For each significant SNP, the minor allele (in unaffected siblings) in both the homozygous and heterozygous states versus the common allele in the homozygous state was examined in the model.

Genotype and allele frequencies for all SNPs identified as significant were calculated in the affected and separately in unaffected siblings (Table 5 [available at <http://aaojournal.org>]). Deviation from Hardy–Weinberg equilibrium was tested on each SNP using the chi-square test. Population-attributable risk (PAR) ($\theta \times [RR - 1]/RR$) was calculated for the significant SNPs that were identified from FBAT and CLR analysis for our 134 matched discordant sibpair data, where the relative risk (RR) is approximated by the odds ratio (OR)²⁶ and θ is the proportion of cases exposed to the factor for each significant SNP in the 10q26 region.

For linkage analysis of the 8 microsatellite markers, identity by state (IBS) scores were calculated from the number of alleles (0, 1, or 2) shared between each pair, the index, and the discordant sibling, for each of the 8 markers. Using heterozygosities for each marker obtained from Map-O-Mat (<http://compugen.rutgers.edu/mapomat>), the expected IBS (null hypothesis of no linkage) was calculated and then compared with the observed IBS values. A goodness-of-fit test was applied to assess the significance of the difference between the observed and expected distributions. Bonferroni correction was applied to the P values of the association tests on microsatellite markers and AMD risk.

Results

We identified 23 variants including deletions. Only the SNPs that had a minor allele frequency of $\geq 5\%$ in the affected and unaffected siblings combined were used for statistical analysis ($n = 19$) (Table 4 [available at <http://aaojournal.org>]). Using FBAT, 6 SNPs showed significant association with AMD risk after applying a Bonferroni correction (Table 6 [available at <http://aaojournal.org>]). Genotype and allele frequencies for each of these SNPs are given in Table 5 (available at <http://aaojournal.org>). No significant deviations from Hardy–Weinberg equilibrium for any of the variants studied were observed in either affected or unaffected siblings, indicating unlikely contamination of our dataset. FBAT demonstrated that the variant most strongly associated with AMD risk was a previously unreported synonymous change in exon 1 of *HTRA1*, triallelic SNP rs2293870 ($P < 10^{-4}$). Additionally, we identified novel significant associations with AMD susceptibility for an intronic deletion in hypothetical locus *LOC387715/ARMS2*, SNP rs10664316 ($P < 10^{-3}$); the *HTRA1* promoter SNP rs2672598 ($P < 10^{-2}$); and another synonymous *HTRA1* SNP, rs1049331 ($P < 10^{-3}$) (Table 4). In agreement with others, FBAT demonstrated that SNPs rs10490924 ($P < 10^{-3}$)

and rs11200638 ($P < 10^{-3}$) were significantly associated with AMD risk, whereas no significant association was observed between neovascular AMD and *PLEKHA1*.^{12,13} Except for SNP rs2293870, all significant SNPs were part of the same haplotype block as depicted in the LD (r^2) plot in Figure 1 (available at <http://aaojournal.org>). The SNP rs10490924 was in high LD with *HTRA1* SNPs rs11200638 and rs1049331 ($r^2 > 0.90$). The intronic SNP rs10664316 was not in high LD with any other SNPs ($r^2 > 0.50$), although we found this small portion of the hypothetical *LOC387715/ARMS2* gene to be conserved in the mouse (http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000166033). Linkage analysis supported our findings of SNP association, as IBS scores calculated for each of the 8 highly heterozygous microsatellite markers analyzed in this region demonstrated that D10S1656 was significantly associated with neovascular AMD ($P < 10^{-15}$) (see “Materials and Methods” and Table 7 [the latter available at <http://aaojournal.org>]).

FBAT demonstrated that 2 haplotypes (h2 and h6) in the 10q26 region were significantly associated with AMD risk (Table 8 [available at <http://aaojournal.org>]). The SNPs rs10490924, rs10664316, rs11200638, rs2672598, and rs1049331 were more strongly associated with increased AMD risk as part of the most significant haplotype, h2 ($P < 10^{-4}$) than when examined individually in FBAT analysis (Table 6).

Multiple CLR analyses were conducted to determine how each of the 6 significant SNPs contributed to the risk of neovascular AMD while adjusting for *CFH* 402H genotype and smoking and, moreover, to examine if interactions existed between each SNP, *CFH*, and/or smoking (Table 9). For each significant SNP, models were created to examine the minor allele in unaffected siblings in both the homozygous and heterozygous states versus having the common allele in the homozygous state. When we included smoking and *CFH* in each model with any of the significant variants, the significance and effect size were not modified with respect to AMD risk. Multiple CLR demonstrated that the most significantly associated variants with increased AMD risk were *HTRA1* SNPs rs11200638 (AA vs. GG: OR, 98.41; confidence interval [CI], 13.45–720.08; $P < 10^{-5}$) (AG vs. GG: OR, 6.05; CI, 2.13–17.21; $P < 10^{-3}$) and rs1049331 (TT vs. CC: OR, 105.52; CI, 14.64–760.5; $P < 10^{-5}$) (TC vs. CC: OR, 5.97; CI, 2.10–16.99; $P < 10^{-3}$). The *HTRA1* SNP rs2672598 conferred a 33-fold reduced risk of AMD homozygously ($P < 10^{-4}$) and 8-fold heterozygously ($P < 10^{-3}$). The minor alleles in the homozygous state, when compared with the common alleles in the homozygous state for SNPs rs10490924, rs11200638, rs1049331, rs2672598, and rs2293870, more strongly influenced AMD risk than the *CFH* Y402H C allele in the homozygous state (vs. TT). As we previously reported¹⁷ and can be seen in our expanded population in Table 9, one C allele for *CFH* Y402H was not significantly associated with neovascular AMD risk ($P > 0.2$).

For each of our significant SNPs in the 10q26 region, there were no interactions between the homozygous or heterozygous genotypes and smoking or between the homozygous and heterozygous genotypes and *CFH* CC genotype.

Another way of looking at the influence of these genetic variants on AMD risk is by looking at the PAR, an estimate of what percentage of cases can be attributed to a particular risk factor. For example, Table 10 shows the PAR for the 6 SNPs significantly associated with

risk of neovascular AMD. Using rs11200638 as an example, it is the A allele that is associated with increased AMD risk (G>A). The presence of 2 A alleles (2 risk alleles) explains 32% of the risk in the entire population (adjusted PAR = 0.32). This is similar in the instance of having at least 1 A allele: 34% of the risk in the entire population is explained (adjusted PAR = 0.34). However, when examining the presence of at least 1 A allele in combination with established risk factors (i.e., *CFH* and smoking) the adjusted PAR % is 82% (adjusted PAR = 0.82). Applying the above to each of the 5 other significantly associated *LOC387715/ARMS2/HTRA1* SNPs (the presence of 1 risk allele for any of these SNPs) shows that the PAR % explains 71% to 82% of risk in the total population when combined with *CFH* and smoking.

We wanted to assess the potential functional effect of the unreported SNPs newly identified as significantly associated with AMD susceptibility. Given that the SNP rs10664316 is located in an intron 4782 base pairs upstream of the first *HTRA1* coding ATG and is weakly conserved in mice, we focused our attention on the highly conserved novel SNPs we identified in the promoter region and exon 1 of *HTRA1* (http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000166033). Using the computer program MapInspector (www.genomatrix.de), we found that the protective allele for the SNP rs2672598 created a binding site for the transcription factor ELK-1. We also found that the *HTRA1* triallelic SNP rs2293870 was located in a binding domain for insulinlike growth factor (IGF) (<http://smart.embl-heidelberg.de> and http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000166033).

The synonymous variants rs1049331 and rs2293870 identified in exon 1 of *HTRA1* did not appear to alter a splice site (http://www.fruitfly.org/seq_tools/splice-instrucs.html).

Discussion

Two of the 4 novel variants that we identified in *HTRA1* have predicted functional effects on the pathophysiology of neovascular AMD. Variant rs2672598 created a binding site for the transcription factor ELK-1, whose activity was reported to be impaired in a patient with a premature form of aging and insulin resistance.²⁷ We hypothesize, then, that if the risk allele of the promoter SNP rs11200638 results in increased expression of *HTRA1*,^{12,13} then the minor allele of rs2672598 might exert a protective effect by enabling the binding of *ELK-1*, which could downregulate the expression of *HTRA1*. On the other hand, the SNP rs2293870 is located in one of the *HTRA1* binding domains for IGFs.²⁸ The replacement of a purine with a pyrimidine (G > C or T) may conceivably alter the proper binding of *HTRA1* for IGF. Further, IGF has been implicated in other ocular conditions characterized by neovascularization, such as diabetic retinopathy and retinopathy of prematurity.²⁹ Therefore, the potential effect of rs2293870 on a regulatory pathway involving *HTRA1* and IGFs has biologic plausibility for AMD. Both variants merit further investigation both in vivo and in vitro. Although we identified 2 other SNPs, rs10664316 and rs1049331 (associated with reduced and increased AMD risk, respectively), that do not appear to have predicted functional effects, this does not preclude them from being biomarkers that in fact could be functionally associated with AMD susceptibility.^{30,31}

In summary, in a population of extremely discordant sibling pairs, we identified unreported variants in the *HTRA1* region that both increase and decrease risk of neovascular AMD. These findings validate previous reports showing that *HTRA1* is the likely candidate gene in the 10q26 region.

Although we identified another novel hypothetical *LOC387715/ARMS2* gene variant, rs10664316, that may ultimately play a role in AMD susceptibility,^{30,31} our newly described variants in *HTRA1* have postulated functional regulatory effects, suggesting possible etiologic mechanisms. Our findings validate that the 10q26 region is more strongly associated with neovascular AMD risk than the 1q32 region in which *CFH* resides.^{12,13,32} Moreover, our results demonstrate that *HTRA1* variants influence risk independent of *CFH* genotype and smoking, supporting the role for *HTRA1* in a distinct pathway underlying AMD pathogenesis.

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Figure 1.

Ensembl single-nucleotide polymorphisms (SNPs) are shown along the 10q26 region encompassing the pleckstrin homology domain– containing protein gene, *LOC387715/ARMS2*, and HtrA serine peptidase 1 gene, illustrating the 3 distinct haplotype blocks that were defined by the confidence intervals, an algorithm proposed by Gabriel et al using Haploview. The linkage disequilibrium (LD) (r^2) between any 2 SNPs is listed in the cross cell. The darker the color, the higher the LD between any 2 SNPs. kb = kilobase.

Table 1

Location of Microsatellite Markers and Single-Nucleotide Polymorphisms (SNPs)

Marker/SNP	Physical Location (Base Pairs)
D10S1690	95984772
D10S1230	122732682
D10S1483	123273561
<i>PLEKHA1</i> rs1045216	124179187
<i>LOC387715/ARMS2</i> rs10490923	124204241
<i>LOC387715/ARMS2</i> rs2736911	124204345
<i>LOC387715/ARMS2</i> rs10490924	124204438
<i>LOC387715/ARMS2</i> rs10664316	124206376
<i>LOC387715/ARMS2</i> rs7088128	124206400
<i>HTRA1</i> rs11200638	124210534
<i>HTRA1</i> –602 from ATG* G>A	124210557
<i>HTRA1</i> –502 from ATG* C>T	124210657
<i>HTRA1</i> rs2672598	124210672
<i>HTRA1</i> rs1049331	124211260
<i>HTRA1</i> rs2293870	124211266
<i>HTRA1</i> rs12267142	124238232
<i>HTRA1</i> rs2239586	124239225
<i>HTRA1</i> rs2672582	124256490
<i>HTRA1</i> intron 5 (del GTTT)	124256969–124256981
<i>HTRA1</i> rs2672583	124257077
<i>HTRA1</i> rs3013206	124257163
<i>HTRA1</i> rs2672585	124258391
<i>HTRA1</i> rs11538140	124261518
<i>HTRA1</i> rs2272599	124261585
<i>HTRA1</i> rs28689095	124261715
<i>HTRA1</i> rs2293871	124263661
D10S587	125178595
D10S1213	125356627
D10S1723	125650839
D10S1656	126091772
D10S1222	129150739

The physical location of each microsatellite marker was determined using Map-O-Mat (<http://compugen.rutgers.edu/mapomat>), and the physical location of each SNP was determined using Ensembl (http://www.ensembl.org/Homo_sapiens/index.html).

* First coding ATG.

Table 2

Subject Characteristics

Population	Mean Age	Range	Standard Deviation	% Male (n/134)
Affected siblings	71.28	48.97–86.38	8.26	45.5% (61)
Unaffected siblings	72.77	41.32–90.86	8.97	39.6% (53)

Table 3

Primers Used

Gene	Region	Forward Primer*	Reverse Primer*
<i>PLEKHA1</i>	Exon 12	CTGACCGTGTCTGACTGCC	CCCCTTATCATCTTTGGCTA
Putative <i>LOC387715/ARMS2</i>	Exon 1	TTGTGTGACGGGAAAAGACA	AAGCACCTGAAGGCTGGTTA
Putative <i>LOC387715/ARMS2</i>	Exon 2	TTGTTACAAAAGGAATGGAATGTC	GGAATGCAGTGACAGAGAGGA
<i>HTRA1</i>	Promoter a	ATGCCACCCACAACAACCTTT	GGTTCTCTCGCTGAGATTCTG
<i>HTRA1</i>	Promoter b	CGGATGCACCAAGATTCTCC	TTCGCGTCCTTCAAACCTAATGG
<i>HTRA1</i>	Exon 1a	GAGGCCCTCCTGCACTCT	CAGGTTGGCGTAGGTGTTG
<i>HTRA1</i>	Exon 1b	GAGTCGCCATGCAGATCC	CGAGCTGGGATGGAGAGA
<i>HTRA1</i>	Exon 2	AAACAAACTTGGGCCATCAG	TTGCTAGTGGCGGTGAAAG
<i>HTRA1</i>	Exon 3	TAGGTGTGTGTGGCTGTTGC	AAGTTTTCCTGAGCCCCCTTC
<i>HTRA1</i>	Exon 4	CGCAGCAAAGGGATGTTAGT	GAATCCACATGGCTTGGTCT
<i>HTRA1</i>	Exon 5	CCAGGCAGGGACATAGATTG	TCAGCAGCCCAGGAGATTGA
<i>HTRA1</i>	Exon 6	GGTGTCTGATGCCTCTCTC	TGCCATGATCAGAGGACAAA
<i>HTRA1</i>	Exon 7	GTCCAGACCAGGATTGAGC	CCAAGGCTAATGACCTGTCC
<i>HTRA1</i>	Exon 8	AGGAGAAGACGGGAACCTGGT	CTCGTGGAGCAAGGACTTTT
<i>HTRA1</i>	Exon 9/3'-UTR	CTGACCCACTGATGGTTTGA	CTATTCCAGCAGCCCAGAGT

* Primers are written in the 5' to 3' direction and were chosen to encompass the entire coding region and flanking intronic sequences using the Primer3 program (<http://primer3.sourceforge.net>).

Table 4

Description of Single-Nucleotide Polymorphisms (SNPs) Analyzed

rs No.	Gene	Region	Nucleotide Change	Amino Acid Change (If Applicable)	Physical Location (Base Pairs)	MAF (Unaffected)	Frequency in Affected
1045216	<i>PLEKHA1</i>	Exon 12	G>A	Thr320Ala	124179187	A = 0.291	A = 0.254
10490923	<i>LOC387715/ARMS2</i>	Exon 1	G>A	Arg3His	124204241	A = 0.108	A = 0.060
2736911	<i>LOC387715/ARMS2</i>	Exon 1	T>C	Arg38End	124204345	C = 0.097	C = 0.086
10490924	<i>LOC387715/ARMS2</i>	Exon 1	G>T	Ala69Ser	124204438	T = 0.328	T = 0.526
10664316	<i>LOC387715/ARMS2</i>	Intron 1	del AT		124206375^124206376	-- = 0.414	-- = 0.269
7088128	<i>LOC387715/ARMS2</i>	Intron 1	A>G		124206400	G = 0.119	G = 0.067
11200638*	<i>HTRA1</i>	Promoter	G>A		124210534	A = 0.317	A = 0.522
	<i>HTRA1</i>	Promoter	G>A		124210557	A = 0.022	A = 0.015
	<i>HTRA1</i>	Promoter	C>T		124210657	T = 0.060	T = 0.049
2672598	<i>HTRA1</i>	Promoter	C>T		124210672	T = 0.496	T = 0.321
1049331	<i>HTRA1</i>	Exon 1	C>T	Ala34Ala	124211260	T = 0.317	T = 0.526
2293870	<i>HTRA1</i>	Exon 1	G>C [†]	Gly36Gly	124211266	C = 0.090	C = 0.086
			G>T	Gly36Gly	124211266	T = 0.317	T = 0.522
12267142*	<i>HTRA1</i>	Intron 1	C>G		124238232	G = 0.022	G = 0.022
2239586	<i>HTRA1</i>	Intron 3	C>T		124239225	T = 0.108	T = 0.078
2239587	<i>HTRA1</i>	Intron 3	G>A		124239299	A = 0.116	A = 0.086
2672582	<i>HTRA1</i>	Intron 4	C>T		124256490	T = 0.429	T = 0.507
	<i>HTRA1</i>	Intron 5	del GTTT		124256973^124256976	-- = 0.392	-- = 0.351
2672583	<i>HTRA1</i>	Intron 5	G>A		124257077	A = 0.422	A = 0.515
3013206*	<i>HTRA1</i>	Intron 5	A>T		124257163	T = 0.045	T = 0.022
2672585	<i>HTRA1</i>	Intron 6	C>G		124258391	G = 0.425	G = 0.507
11538140*	<i>HTRA1</i>	Exon 8	C>T	Asp407Asp	124261518	T = 0.004	T = 0.004
2272599	<i>HTRA1</i>	Intron 8	A>G		124261585	G = 0.474	G = 0.545
2293871	<i>HTRA1</i>	Intron 8	C>T		124263661	T = 0.138	T = 0.101

MAF = minor allele frequency.

The physical location of each SNP was determined using Ensembl (http://www.ensembl.org/Homo_sapiens/index.html).

* Variants/SNPs excluded from statistical analysis because MAF did not meet the criteria of 5% in both affected and unaffected siblings combined.

⁷The most minor of the 3 alleles

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Table 5

Genotype and Allele Frequencies

	<u>Affected Siblings (Index Cases)</u>		<u>Unaffected Siblings</u>	
	Frequency (%)	N	Frequency (%)	N
<i>LOC387715/ARMS2</i>				
rs10490924				
Genotype				
TT	33.58	45	16.42	22
TG	38.06	51	32.84	44
GG	28.36	38	50.75	68
Total		134	134	
Allele				
T	52.61	141	32.84	88
G	47.39	127	67.16	180
Total		268		268
rs10664316				
Genotype				
--	11.19	15	18.66	25
--AT	31.34	42	45.52	61
AT	57.46	77	35.82	48
Total		134		134
Allele				
--	26.87	72	41.42	111
AT	73.13	196	58.58	157
Total		268		268
<i>HTRA1</i>				
rs11200638				
Genotype				
AA	32.09	43	15.67	21
AG	40.30	54	32.09	43
GG	27.61	37	52.2	70
Total		134		134
Allele				
A	52.24	140	31.72	85
G	47.76	128	68.28	183
Total		268		268
rs2672598				
Genotype				
TT	11.94	16	26.12	35
TC	40.30	54	47.01	63
CC	47.76	64	26.87	36
Total		134		134

		<u>Affected Siblings (Index Cases)</u>		<u>Unaffected Siblings</u>	
		Frequency (%)	N	Frequency (%)	N
Allele					
T	32.09	86	49.63	133	
C	67.91	182	50.37	135	
Total		268		268	
rs1049331					
Genotype					
TT	32.84	44	15.67	21	
TC	39.55	53	32.09	43	
CC	27.61	37	52.24	70	
Total		134		134	
Allele					
T	52.61	141	31.72	85	
C	47.39	127	68.2	183	
Total		268		268	
rs2293870					
Genotype					
CC	0.75	1	2.24	3	
TT	31.34	42	15.67	21	
CG	6.72	9	9.70	13	
TG	32.84	44	28.36	38	
CT	8.96	12	3.73	5	
GG	19.41	26	40.30	54	
Total		134		134	
Allele					
C	8.58	23	8.96	24	
T	52.23	140	31.72	85	
G	39.18	105	59.33	159	
Total		268		268	

Table 6
Single-Marker Analysis Results from the FBAT Program Assuming an Additive Genetic Model

SNP	No. of Informative Families	Variance (S)	Z Value	Pointwise P Value	Familywise P Value*
<i>PLEKHA1</i> rs1045216	16	16,000	0.500	0.6171	1.0
<i>LOC387715/ARMS2</i> rs10490923	3	†	†	†	†
<i>LOC387715/ARMS2</i> rs2736911	2	†	†	†	†
<i>LOC387715/ARMS2</i> rs10490924	31	31,000	4.131	0.00036	0.001
<i>LOC387715/ARMS2</i> rs10664316	47	47,000	4.230	0.000023	0.0007
<i>LOC387715/ARMS2</i> rs7088128	25	25,000	2.600	0.009322	0.27
<i>HTRA1</i> rs11200638	28	28,000	4.158	0.000032	0.0009
<i>HTRA1</i> –502 (from ATG [‡])	2	†	†	†	†
<i>HTRA1</i> rs2672598	27	27,000	3.657	0.000256	0.007
<i>HTRA1</i> rs1049331	29	29,000	4.271	0.000019	0.0006
<i>HTRA1</i> rs2293870 (T vs. G/C)	61	26,500	4.662	3.13×10 ⁻⁶	0.00009
<i>HTRA1</i> rs2293870 (G vs. T/C)	65	26,000	5.295	1.19×10 ⁻⁶	0.00003
<i>HTRA1</i> rs2239586	2	†	†	†	†
<i>HTRA1</i> rs2239587	2	†	†	†	†
<i>HTRA1</i> rs2672582	29	29,000	1.671	0.0947	1.0
<i>HTRA1</i> intron 5 (del GTTT)	41	41,000	0.781	0.4349	1.0
<i>HTRA1</i> rs2672583	29	29,000	2.043	0.0411	1.0
<i>HTRA1</i> rs2672585	60	19,500	2.491	0.0127	0.37
<i>HTRA1</i> rs2272599	35	35,000	1.859	0.0630	1.83
<i>HTRA1</i> rs2293871	4	4,000	2.000	0.0455	1.32

SNP = single-nucleotide polymorphism.

Minor allele frequency is 5% in both affected and unaffected siblings combined.

* Applied Bonferroni correction on 29 tests.

† No. of informative families is less than 4, no statistics are available

‡ First coding.

Table 7

Microsatellite Marker Results

	D10S1213	D10S1656*	D10S1723	D10S587	D10S1690	D10S1230	D10S1483	D10S1222
No. of 0s [†]	21	51	21	20	4	10	10	7
No. of 1s [†]	67	55	71	60	37	44	48	59
No. of 2s [†]	42	24	41	51	30	32	38	35
Total	130	130	133	131	71	86	96	101
No. of NAs	4	4	1	3	63	48	38	33
Heterozygosity	0.827	0.750	0.884	0.793	0.650	0.740	0.830	0.730
χ^2	0.480	73.261	1.403	0.360	2.902	0.781	3.429	7.072
P value	0.787	1.23×10^{-16}	0.496	0.835	0.234	0.677	0.180	0.029
P value [‡]	1	9.88×10^{-16}	1	1	1	1	1	0.223

NA = not applicable.

* Located 1.8 megabases from the end of the HtrA serine peptidase 1 gene.

[†] Number of alleles (0, 1, or 2) shared between the sibling pair.

[‡] Adjusted values using the Bonferroni correction on 8 tests.

Table 8

Haplotype Analysis Results from the FBAT

Order	LOC387715/ARMS2 rs10490923	LOC387715/ARMS2 rs2736911	LOC387715/ARMS2 rs10490924	LOC387715/ARMS2 rs10664316	LOC387715/ARMS2 rs7088128	HTRAI rs11200638	HTRAI-502 (from ATG [*]) C>T	HTRAI rs2672598	HTRAI rs1049331	No. of Informative Families	Frequency	P Value [†]
h1	G	C	G	—	A	G	C	C	C	47	0.347	0.1113
h2	G	C	T	AT	A	A	C	C	T	27	0.235	0.00002
h3	G	C	G	AT	A	G	C	C	C	24	0.104	0.2304
h4	G	C	G	—	A	G	C	T	C	16	0.104	0.1433
h5	G	C	G	AT	G	G	C	C	C	15	0.078	0.4555
h6	G	C	G	—	G	G	C	T	C	10	0.060	0.0010

h*n* = haplotype *n*.

Estimated haplotypes with allele frequency > 0.05 were listed and tested for association. When considering all possible haplotypes together, the resulting

^{*} First coding ATG.

[†] Results based on 100 000 permutations.

P value from 100 000 permutations was 0.00006.

Table 9

Results of Multiple Conditional Logistic Regression Analysis Showing Significant Single-Nucleotide Polymorphisms

Risk Factor	Referent	Odds Ratio (95% CI)	P Value
<i>LOC387715/ARMS2</i> rs10490924 “T” is rare allele			
rs10490924 TT	GG	61.91 (10.89–352.01)	0.0000033
rs10490924 TG	GG	5.32 (1.82–15.52)	0.0022
Smoking 10 pack-years	<10 pack-years	3.81 (1.84–7.91)	0.0003
<i>CFH</i> CC	TT	18.13 (4.20–78.22)	0.0001
<i>CFH</i> TC	TT	1.78 (0.72–4.40)	0.2089
<i>LOC387715/ARMS2</i> rs10664316 “del AT” is rare allele			
rs10664316 del AT/del AT	AT/AT	0.09 (0.02–0.36)	0.0007
rs10664316 del AT/AT	AT/AT	0.30 (0.13–0.72)	0.0069
Smoking 10 pack-years	<10 pack-years	3.00 (1.58–5.70)	0.0008
<i>CFH</i> CC	TT	12.64 (3.26–49.04)	0.0002
<i>CFH</i> TC	TT	1.56 (0.68–3.58)	0.2998
<i>HTRA1</i> rs11200638 “A” is rare allele			
rs11200638 AA	GG	98.41 (13.45–720.08)	0.0000062
rs11200638 AG	GG	6.05 (2.13–17.21)	0.0007
Smoking 10 pack-years	<10 pack-years	4.08 (1.93–8.63)	0.0002
<i>CFH</i> CC	TT	19.59 (4.36–88.03)	0.0001
<i>CFH</i> TC	TT	1.69 (0.67–4.26)	0.2687
<i>HTRA1</i> rs2672598 “T” is rare allele			
rs2672598 TT	CC	0.03 (0.01–0.14)	0.000013
rs2672598 TC	CC	0.12 (0.04–0.39)	0.0004
Smoking 10 pack-years	<10 pack-years	3.48 (1.73–7.01)	0.0005
<i>CFH</i> CC	TT	10.34 (2.76–38.67)	0.0005
<i>CFH</i> TC	TT	1.69 (0.70–4.06)	0.2428
<i>HTRA1</i> rs1049331 “T” is rare allele			
rs1049331 TT	CC	105.52 (14.64–760.50)	0.0000038
rs1049331 TC	CC	5.97 (2.10–16.99)	0.0008
Smoking 10 pack-years	<10 pack-years	4.09 (1.93–8.66)	0.0002
<i>CFH</i> CC	TT	19.29 (4.28–86.94)	0.0001
<i>CFH</i> TC	TT	1.67 (0.66–4.20)	0.2754
<i>HTRA1</i> rs2293870 “T” and “C” are rare alleles			
rs2293870 CC, CT or TT	GG	25.97 (6.32–106.66)	0.0000062
rs2293870 CG or TG	GG	5.89 (1.96–17.71)	0.0016
Smoking 10 pack-years	<10 pack-years	3.31 (1.66–6.60)	0.0006
<i>CFH</i> CC	TT	13.94 (3.37–57.67)	0.0003
<i>CFH</i> TC	TT	1.58 (0.64–3.90)	0.3227

CFH = complement factor H gene; CI = confidence interval.

Table 10

Population-Attributable Risk (PAR) Table

Exposure	Relative Risk	Frequency in Cases, θ	Adjusted PAR (95% Confidence Interval)
<i>LOC387715/ARMS2</i> rs10490924			
TT versus GG	61.9	0.34	0.33 (0.23–0.42)
TG versus GG	5.3	0.39	0.32 (0.21–0.41)
Smoking (<10 vs. 10 pack-years)	3.8	0.54	0.40 (0.30–0.48)
Smoking 10 pack-years or T allele *	4.0	0.87	0.65 (0.60–0.70)
<i>CFH</i> CC versus TT	18.1	0.34	0.32 (0.22–0.41)
Smoking 10 pack-years or T allele or <i>CFH</i> CC *	10.3	0.90	0.81 (0.78–0.84)
<i>LOC387715/ARMS2</i> rs10664316			
AT/AT versus del AT/del AT	11.5	0.57	0.52 (0.45–0.59)
AT/AT versus del AT/AT	3.5	0.31	0.22 (0.10–0.33)
Smoking (<10 vs. 10 pack-years)	3.0	0.54	0.36 (0.26–0.45)
Smoking 10 pack-years or AT allele *	2.5	0.94	0.56 (0.50–0.62)
<i>CFH</i> CC versus TT	12.6	0.34	0.31 (0.21–0.41)
Smoking 10 pack-years or AT/AT (no del allele) or <i>CFH</i> CC *	6.0	0.85	0.71 (0.66–0.75)
<i>HTRA1</i> rs11200638			
AA versus GG	98.4	0.32	0.32 (0.21–0.41)
AG versus GG	6.1	0.41	0.34 (0.24–0.43)
Smoking (<10 vs. 10 pack-years)	4.1	0.54	0.41 (0.32–0.49)
Smoking 10 pack-years or A allele *	4.1	0.87	0.66 (0.60–0.70)
<i>CFH</i> CC versus TT	19.6	0.34	0.32 (0.22–0.41)
Smoking 10 pack-years or A allele or <i>CFH</i> CC *	10.7	0.91	0.82 (0.80–0.85)
<i>HTRA1</i> rs2672598			
CC versus TT	36.5	0.48	0.46 (0.38–0.54)
TC versus TT	4.3	0.41	0.32 (0.21–0.41)
Smoking (<10 vs. 10 pack-years)	3.5	0.54	0.39 (0.29–0.47)
Smoking 10 pack-years or C allele *	3.5	0.94	0.67 (0.62–0.72)
<i>CFH</i> CC versus TT	10.3	0.34	0.30 (0.20–0.40)
Smoking 10 pack-years or CC (no T allele) or <i>CFH</i> CC *	8.0	0.86	0.75 (0.71–0.79)
<i>HTRA1</i> rs1049331			
TT versus CC	105.5	0.33	0.33 (0.22–0.42)
TC versus CC	6.0	0.40	0.33 (0.23–0.42)
Smoking (<10 vs. 10 pack-years)	4.1	0.54	0.41 (0.32–0.49)
Smoking 10 pack-years or T allele *	4.1	0.87	0.66 (0.60–0.70)
<i>CFH</i> CC versus TT	19.3	0.34	0.32 (0.22–0.41)
Smoking 10 pack-years or T allele or <i>CFH</i> CC *	10.7	0.91	0.82 (0.80–0.85)
<i>HTRA1</i> rs2293870			
CC, CT, or TT versus GG	26.0	0.41	0.39 (0.30–0.48)

Exposure	Relative Risk	Frequency in Cases, θ	Adjusted PAR (95% Confidence Interval)
CG or TG versus GG	5.9	0.40	0.33 (0.23–0.42)
Smoking (<10 vs. 10 pack-years)	3.3	0.54	0.38 (0.28–0.46)
Smoking 10 pack-years or C or T allele *	3.4	0.90	0.64 (0.58–0.68)
<i>CFH</i> CC versus TT	13.9	0.34	0.31 (0.21–0.41)
Smoking 10 pack-years or C or T allele or <i>CFH</i> CC *	8.0	0.94	0.82 (0.78–0.86)

CFH = complement factor H gene.

Relative risk is estimated by conditional logistic regression adjusting for other factors.

* This value is less than the sum of the adjusted PARs, because these risk factors are not mutually exclusive, and the relative risk used here is not adjusting for other factors.