Common variation in the **SERPING1** gene is not associated with age-related macular degeneration in two independent groups of subjects

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**Purpose:** Common genetic variation in the complement component 1 inhibitor gene (**SERPING1**) was recently reported to increase the risk of developing age-related macular degeneration (AMD). This study was performed to replicate the association between **SERPING1** and AMD.

**Methods:** Seven single nucleotide polymorphisms (SNPs) tagging common haplotypes across **SERPING1** were genotyped on 786 (The Mayo Clinic) subjects and the association with AMD studied using single SNP and haplotype association analyses. The SNP in intron 6 (rs2511989) previously reported to increase the risk of AMD was studied in an additional 1,541 subjects from the Age-Related Eye Disease Study (AREDS). Association with specific subtypes of AMD and interaction with four other loci: complement factor H (**CFH**), age-related maculopathy susceptibility 2 (**ARMS2/LOC387715**), High Temperature Requirement Factor A1 (**HTRA1**), complement factor B/complement component 2 (**CFB/C2**), and complement component 3 (**C3**) involved in AMD was explored.

**Results:** The seven tag-SNPs were not associated with AMD in the Mayo subjects (p=0.13–0.70) and rs2511989 was also not associated with AMD in the Mayo or AREDS subjects (p=0.44–0.45). Evaluation of haplotypes across **SERPING1** did not reveal association with AMD (p=0.14–0.97). SNPs were not associated with AMD subtypes (early, geographic atrophy, or exudation). No interaction with other AMD risk variants was observed.

**Conclusions:** We were unable to replicate the reported association between **SERPING1** and AMD in two independent groups of subjects.

Age related macular degeneration (AMD) is a leading cause of irreversible loss of vision in older individuals [1]. Both genetic and cell biologic analyses support a role for dysregulation of innate immunity in the pathogenesis of AMD. Extensive deposition of innate immunity proteins at the level of Bruch’s membrane [2,3] in AMD patients has been consistently observed. Genetic variants in complement pathway genes including complement factor H (**CFH**) [4-6], complement factor B (**CFB**) [7], and complement component 3 (**C3**) [8-11] are established risks for developing AMD. The association between AMD and genetic variation in these loci has been extensively replicated as recently reviewed [12]. Many other common genetic variants have been proposed for AMD. However, review of a partial list shows that they do not yet have sufficient support due to the absence of replication studies [13-20] or failure to consistently replicate the genetic association in independent groups of subjects [21-29]. Thus, at this time there is strong evidence for the involvement of genetic variation in genes of the alternative pathway of complement and one additional locus (**LOC387715/High Temperature Requirement Factor A1 [HTRA1]**) on chromosome 10q26 [30,31].

Recently, there has been interest in exploring the genes involved in classical complement activation initiated by antibody-antigen interactions. Evidence for a possible role of the classical complement pathway comes from the observation of anti-retinal antibodies in patients with AMD [32-35], animal models with AMD-like pathology [36], and expression of these proteins in the aging retina and retinal pigment epithelium (RPE) [37].

The complement component 1 (C1) inhibitor is a key regulator of the classical pathway and has been reported to down-regulate the alternative pathway in vitro by binding to C3b and inhibiting binding of complement factor B to C3b [38]. The C1 inhibitor has sequence homology with serine protease inhibitors (SERPIN) and inhibits activation of the classical and lectin complement pathways by inhibiting the protease activity of complement component 1 r subcomponent (C1r), complement component 1 s subcomponent (C1s), and mannan-binding lectin serine peptidase 2 (MASP-2) [39,40]. The C1 inhibitor also regulates vascular permeability by inhibiting proteases that generate
bradykinin [40], as manifested when deficiency of C1 inhibitor results in hereditary angioedema [41-43]. Thus, C1 inhibitor is an excellent candidate gene for involvement in AMD and retinal aging. Since the discovery of the association between AMD and variants in CFH, the systematic study of other genes regulating or involved in the alternative pathway of complement activation has been productive (e.g., the study of CFB/C2, C3 [7-11]). The systematic study of other genes involved in innate immunity is ongoing in several laboratories. A recent study reported a protective effect on AMD for the minor allele of a SNP (rs2511989) within intron 6 of the SERPING1 gene encoding the C1 inhibitor [13]. The purpose of this study was to replicate the association between SERPING1 and AMD in two independent groups of subjects. We performed a systematic analysis of all common haplotypes (ancestral segments of DNA inherited as a block in a population) across the SERPING1 locus and identified no evidence for association with AMD.

METHODS

Subjects: The study was approved by the institutional review board of the Mayo Clinic (Rochester, MN) and written informed consent was obtained from all subjects. The Mayo subjects were composed of 786 Caucasian individuals (476 AMD cases, 310 controls without AMD). Subjects and cases were obtained from the same eye clinics of participating physicians. Diagnosis was determined by review of fundus photographs as described previously [4,44,45]. Briefly, all subjects diagnosed with AMD had large drusen (≥125 microns) with sufficient drusen area to fill a 700 micron circle or more advanced findings. Controls had 5 or fewer hard drusen (<63 microns) without pigment changes or more advanced findings. Geographic atrophy and exudation were defined using the Wisconsin age-related maculopathy grading system [46]. The Mayo subjects have been graded multiple independent times by Dr. Edwards and were recently re-

graded by Dr. Park. Replication studies were performed on 1,541 Caucasian subjects (1,241 with AMD and 300 controls without AMD) from the Age-Related Eye Disease Study (AREDS) that were graded as reported previously [47]. The final AREDS phenotype grade was used. All control grades (controls and control questionable 1–4) were treated as controls. Early AMD consisted of all grades of large drusen (large drusen and large drusen questionable 1–3). Review of genotype frequencies for the control questionable and large drusen questionable AREDS subjects at known AMD risk variants showed that the questionable groups closely matched their assigned group (e.g., for the C3 SNP rs2230199, genotype frequencies were within 1% when the questionable control or large drusen categories were excluded). Further, the maximal difference in genotype frequencies for the SERPING1 SNP rs2511989 was 0.03.

Advanced AMD AREDS subjects consisted of all advanced AMD grades (questionable advanced, neovascular, geographic atrophy, and both neovascular and geographic atrophy). Mayo or AREDS subjects with both geographic atrophy and exudative AMD (category “Both”) were included in the geographic atrophy group as described in the Methods.

TABLE 1. DEMOGRAPHIC AND PHENOTYPIC FEATURES OF THE MAYO AND AREDS SUBJECTS

| Category              | Mayo subjects | AREDS subjects |
|-----------------------|---------------|----------------|
|                       | Number | Age (Mean±SD) | Male:Female ratio | Number | Age (Mean±SD) | Male:Female ratio |
| Control subjects      | 310    | 69.5±8.2      | 0.83              | 300    | 77.6±4.3      | 0.79              |
| AMD subjects*         | 476    | 76.9±9.6      | 0.55              | 1241   | 79.9±5.1      | 0.68              |
| Early AMD             | 218    | 73.7±10.3     | 0.46              | 583    | 79.0±4.9      | 0.65              |
| Exudation             | 161    | 79.2±8.7      | 0.59              | 324    | 80.6±5.0      | 0.76              |
| Geographic atrophy**  | 97     | 80.2±6.6      | 0.70              | 247    | 80.8±5.3      | 0.65              |

*Eighty-seven AREDS subjects with the “questionable advanced age-related macular degeneration (AMD)” grade were used as described in Methods, but are not included in this table. **29 Mayo subjects and 85 AREDS subjects who had both geographic atrophy and exudative AMD (category “Both”) were included in the geographic atrophy group as described in the Methods.

Abbreviations: SD is standard deviation.
Selection of tag and functional SNPs: SNPs genotyped in the international haplotype map (HapMap) [48] were used to select tag SNPs. SNPs across SERPING1 including 5 kb up and downstream were evaluated for linkage disequilibrium (LD) using ldSelect [49] and a custom algorithm for tag-SNP selection was developed so that a single tag SNP would be selected for each LD bin. Only those SNPs deemed candidate tag-SNPs by ldSelect with a minor allele frequency (MAF) of greater than or equal to 0.05 were considered for further selection. Tag-SNPs were ultimately selected based upon a functional ranking system wherein non-synonymous coding SNPs were preferentially selected among the tag-SNP candidates in each LD bin, followed by synonymous coding SNPs, SNPs from 5′ untranslated regions (UTRs), SNPs from 3′ UTRs, and finally SNPs from intronic regions. Seven SNPs were required to tag the SERPING1 haplotypes and were selected for genotyping.

Genotyping: The seven tag-SNPs were genotyped on genomic DNA extracted from peripheral blood leukocytes using TaqMan assays. Clustering of genotypes was inspected for separation between genotypes and accuracy of genotype calls. Quality control for genotype call was assessed by concordance for the control CEPH trio DNA replicates and two sample replicates within each 96 well plate. Randomly selected cases (25 subjects) and controls (25 subjects) were subjected to bi-directional DNA sequencing to determine the accuracy of genotyping with rs2511989 in a masked fashion. Statistics: Upon receipt of genotype intensities and processing as described above, all SNPs were noted to be in Hardy–Weinberg equilibrium. Single SNP analyses on genotype distributions were performed in SAS version 8 (SAS Institute, Cary, NC) using logistic regression assuming a log-additive genetic model where SNPs were coded as 0, 1, or 2 for the number of minor alleles and the corresponding p-values were calculated based on the score test statistics. Fisher’s exact tests were also performed on genotype distributions. Haplotype analyses were completed using the score test that consisted of the seven SNPs across SERPING1. We also performed haplotype analysis with a 3-SNP-sliding-window approach, as implemented in haplo.stats [50] using R statistical software. Interaction effect between SERPING1 SNPs and smoking status (defined as ever, never) on AMD status was evaluated under a log-additive model with logistic regression using two main effect terms (genotype for each SNP and smoking status) and their interaction term, and assessed by a likelihood ratio test. We also evaluated interaction between SERPING1 SNPs and other major genetics risks for AMD including complement factor H (CFH, Y402H), LOC387715/HTRAI (tagging SNP A69S), CFB/C2 (CFB, L9H; C2, rs547154), and C3 (R102G and P314L). To investigate the effect of the seven SNPs of SERPING1 including rs2511989 on AMD subtypes, likelihood ratio tests were performed considering controls as baseline. Haplotype analyses for AMD subtypes were also performed using a likelihood ratio test. Age is confounded with diagnosis (i.e., the cases are older than the controls), thus correction for age might reduce any detectable genetic effect and was not employed. Nominal p-values are reported.

RESULTS

Association between individual SNPs and AMD in the Mayo subjects: None of the seven SNPs tagging SERPING1 haplotypes (including rs2511989) were associated with AMD subjects compared to controls without AMD (Table 2). Because of the previously reported association between rs2511989 and AMD, we sought to determine the accuracy of genotyping using DNA sequencing. The genotype for 50 subjects determined by DNA sequencing was in complete agreement with the TaqMan assay genotype calls. Thus, the absence of association could not be explained by inaccurate genotyping.

Linkage disequilibrium: To verify that our genotyping results were similar to other Caucasian populations genotyped across SERPING1, we compared the pattern of linkage disequilibrium (LD) in the Mayo subjects to the international HapMap Caucasian subjects using Haploview [48,51]. Moderate to high LD was observed across the SERPING1 locus and a similar pattern was present in the Mayo subjects and HapMap Caucasian subjects (Figure 1). The similar pattern of LD further supports the accuracy of genotyping of all seven SNPs in the Mayo subjects.

Haplotype studies: Comparison of haplotypes across the SERPING1 locus in the Mayo subjects and the international HapMap Caucasian subjects showed a similar distribution of haplotypes (Table 3). Further, no haplotype was associated with AMD in the Mayo subjects (p=0.14–0.97, Table 3). A 3-SNP sliding window analysis did not reveal association between the SERPING1 haplotypes and AMD (p=0.13–0.67).

Replication study: Given the demonstrated accuracy of genotyping, we sought to determine if the absence of association could be due to a type II error arising in the Mayo subject group. The most highly associated variant in the previous report (rs2511989) was genotyped on 1,541 AREDS subjects (Table 4) and no association with AMD was observed (p=0.45).

AMD subtype analysis: To determine if the absence of association between AMD and rs2511989 could be due to differences in the distribution of AMD subtypes in the different subject groups, we stratified the AMD subjects into early AMD, primary geographic atrophy, and exudation. Subgroup analysis using the seven tag-SNPs genotyped on the Mayo subjects revealed no evidence for association with any AMD subtype (early AMD, p=0.43–0.88; geographic atrophy, p=0.13–0.96; exudation, p=0.05–0.66). Similarly, no association between rs2511989 genotypes and AMD subtypes were observed in the AREDS subjects (p=0.17–0.97). No
## Table 2. Distribution of genotypes for the seven tag-SNPs across SERPING1 and their association with AMD in the Mayo subjects

| SNP       | Function | Location | Minor allele frequency | Hardy–Weinberg equilibrium (p-value) | Genotype | Number AMD Cases (%) | Number controls (%) | Fisher p-value | Log-additive p-value |
|-----------|----------|----------|------------------------|--------------------------------------|----------|----------------------|--------------------|---------------|----------------------|
| rs2509897 | 2.4 K upstream | 57119193 | 0.36                   | 0.87                                 | GG       | 197 (42)             | 122 (39)           | 0.66          | 0.52                 |
|           |          |          |                        |                                      | GC       | 204 (44)             | 146 (47)           | 0.52          |                      |
|           |          |          |                        |                                      | CC       | 64 (14)              | 42 (14)            | 0.52          |                      |
| rs2511990 | 2.3 K upstream | 57119284 | 0.5                    | 0.19                                 | TT       | 126 (27)             | 80 (26)            | 0.72          | 0.51                 |
|           |          |          |                        |                                      | TC       | 223 (48)             | 141 (46)           | 0.52          |                      |
|           |          |          |                        |                                      | CC       | 116 (25)             | 84 (28)            | 0.52          |                      |
| rs3758919 | 2.2 K upstream | 57119379 | 0.11                   | 0.92                                 | CC       | 382 (81)             | 236 (77)           | 0.24          | 0.13                 |
|           |          |          |                        |                                      | CT       | 80 (17)              | 67 (22)            | 0.52          |                      |
|           |          |          |                        |                                      | TT       | 7 (1)                | 5 (2)              | 0.52          |                      |
| rs1005511 | Intron 2 | 57123232 | 0.33                   | 0.26                                 | GG       | 208 (46)             | 124 (43)           | 0.43          | 0.7                  |
|           |          |          |                        |                                      | GA       | 194 (43)             | 137 (47)           | 0.52          |                      |
|           |          |          |                        |                                      | AA       | 52 (11)              | 28 (10)            | 0.52          |                      |
| rs2508443 | Intron 6 | 57134568 | 0.4                    | 0.37                                 | GG       | 180 (38)             | 104 (34)           | 0.27          | 0.47                 |
|           |          |          |                        |                                      | GT       | 211 (45)             | 157 (51)           | 0.52          |                      |
|           |          |          |                        |                                      | TT       | 78 (17)              | 48 (16)            | 0.52          |                      |
| rs2511989 | Intron 6 | 57134901 | 0.4                    | 0.27                                 | GG       | 179 (38)             | 103 (33)           | 0.27          | 0.44                 |
|           |          |          |                        |                                      | GA       | 211 (45)             | 157 (51)           | 0.52          |                      |
|           |          |          |                        |                                      | AA       | 80 (17)              | 50 (16)            | 0.52          |                      |
| rs4926    | V480M (Exon 8) | 57138565 | 0.27                   | 0.52                                 | GG       | 252 (54)             | 167 (54)           | 0.14          | 0.36                 |
|           |          |          |                        |                                      | GA       | 172 (37)             | 124 (40)           | 0.52          |                      |
|           |          |          |                        |                                      | AA       | 47 (10)              | 19 (6)             | 0.52          |                      |

The seven single-nucleotide polymorphisms (SNPs) were tested for association with age-related macular degeneration (AMD) in the Mayo subjects and none was observed.
haplotype was consistently associated with any AMD subtype (p=0.09–0.98).

Interaction with other genetic risks for AMD: We further sought to determine if the absence of an effect of rs2511989 on AMD risk might be explained by differences between modifiable risk factors and other genetic risks for AMD in the Mayo subjects. We observed no interaction between smoking categorized as ever or never and \textit{SERPING1} SNPs using logistic regression (p=0.25–0.52). We also found no interaction (p=0.68–0.98) between rs2511989 and other major genetics risks for AMD including complement factor H (\textit{CFH}, Y402H), \textit{ARMS2/HTRA1} (tagging SNP A69S), complement components \textit{CFB}/\textit{C2} (\textit{CFB}, L9H; \textit{C2}, rs547154), and \textit{C3} (R102G and P314L).

**DISCUSSION**

We were unable to replicate the previously reported association between \textit{SERPING1} and AMD using two independent groups of subjects [13]. Our study comprehensively assessed common variation in \textit{SERPING1} and specifically the SNP (rs2511989) most highly associated with AMD in the previous study [13].

The previous study observed several SNPs (rs2244169, rs2511990, rs2509897, rs2511989, rs2511988, and rs1005510) across \textit{SERPING1} that were associated with AMD [13]. We genotyped three of these SNP (rs2509897, rs2511990, and rs2511989) and observed no association with AMD. Although some investigators have felt that observing association from SNPs near the initial disease associated SNP provides additional support for the
assumption, we have observed otherwise [11]. This situation arises secondary to linkage disequilibrium and supports accurate genotyping, but does not protect against a type I error [11].

We recognize that a particular group of subjects may miss a valid association (type II error) or show false association (type I error). Several causes for such observations exist including invalid genotyping, population stratification, non-random sampling of the underlying population, and differences in non-randomized features of the cases and controls such as disease subtypes. For these reasons, we validated the genotyping with a second independent assay, studied two independent populations, and extensively studied the disease subgroups and similarities between our Caucasian populations and those genotyped elsewhere (HapMap). All of these analyses supported the validity of our study and failed to provide evidence for an effect of SERPING1 SNPs on AMD. Genotyping of additional groups of subjects will be required to determine if SERPING1 SNPs are associated with AMD in selected populations. Nonetheless, a major effect on AMD is unlikely.

Genotyping errors can reduce power and are thus of particular concern in a study that fails to replicate a previous observation. The previous study used an Illumina BeadChip platform in one group of subjects and an assay on demand TaqMan method in a second group of independent subjects [13] for genotyping rs2511989 (Table 4). Eight additional SNPs were genotyped using a third (KBioscience, Hoddesdon, UK) platform. Thus, genotyping errors seem unlikely to have caused a type I error in the previous report. We employed the same genotyping assay (TaqMan, Foster City, CA) used on one of their subject groups on both of our groups of subjects and performed DNA sequencing to confirm genotyping results. Genotyping problems seem unlikely to explain the differences between the studies.

SERPING1 is expressed in the neural retina, RPE, and choroid of humans [13] and is likely to play a role in regulating the complement system in the eye. Mutations in SERPING1 cause hereditary angioedema, but there is no known phenotypic overlap with AMD such as had been established with atypical hemolytic uremic syndrome or glomerulonephritis arising in patients with mutations in CFH [52]. In summary, we were unable to replicate the association between genetic variation in SERPING1 and AMD in two large and well characterized Caucasian subject groups and no compelling biologic evidence is available to support a role for the C1 inhibitor in AMD at this time.

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