Analysis of the Y402H Variant of the Complement Factor H Gene in Age-Related Macular Degeneration

Paul N. Baird, F. M. Amirul Islam, Andrea J. Richardson, Melinda Cain, Nicola Hunt, and Robyn Guymer

PURPOSE. Recent studies in U.S. populations have indicated that the Y402H variant of the complement factor H (CFH) gene contains a major risk susceptibility allele for age-related macular degeneration (AMD). This study was conducted to ascertain whether this is also true in a non-U.S. population and also whether the at-risk allele is associated with the clinical phenotype of disease and the age at diagnosis.

METHODS. Two hundred thirty-six unrelated individuals with AMD and 144 unrelated but ethnically matched control subjects were recruited and examined. All subjects completed a standard questionnaire, were given a fundus examination, and provided a blood sample for DNA extraction. Alleles of Y402H in the CFH gene were determined by use of a MALDI-TOF-based approach followed by statistical analysis.

RESULTS. Individuals with AMD who had at least one copy of the C allele of Y402H had an increased risk of disease (odds ratio [OR] 2.98; 95% confidence interval [CI] 1.81–4.93) compared with cases with the T allele. On subgroup analysis, this risk was found to be most significant in individuals with neovascular disease (OR 4.34; 95% CI 1.94, 9.71). In addition, individuals with neovascular disease who were homozygous CC presented with a significant 7.0-year earlier age at diagnosis relative to those individuals who were homozygous TT. The population-attributable risk for the C allele ranged between 47% to 69%, depending on the AMD disease subtype.

CONCLUSIONS. The C allele of Y402H represents a significant risk factor in individuals with AMD, and this effect is most pronounced in individuals with neovascular disease. (Invest Ophthalmol Vis Sci. 2006;47:4194–4198) DOI:10.1167/iovs.05-1285

Age-related macular degeneration (AMD; MIM 603075 [Mendelian Inheritance in Man; National Center for Biotechnology Information, Bethesda, MD]) is the leading cause of vision loss in Western societies, with the severe vision-threatening complications of geographic atrophy (GA) and choroidal neovascularization (CNV) accounting for nearly 50% of all blindness in the Western world. AMD is very much age dependent, with prevalence of disease increasing from 0.85% in the 65- to 74-year age group, to 4.59% between 75 and 84 years, and to 13.05% in the over-85 age group (pooled data from three studies). A genetic contribution to AMD has been well established through the use of family and twin studies. Genetic linkage studies of AMD have so far identified nine putative loci across the human genome through the use of genome-wide scans, with the two most promising linkage regions occurring on the long arms of chromosomes 1 and 10.

The ARMD1 gene locus at 1q31 was the first linkage region identified. Additional genome-wide scans of multiplex AMD families have replicated linkage to this region and it has been estimated that between 15% and 40% of AMD families segregate with a disease gene in this region. Further analysis of the ARMD1 region identified the Gln5345Arg allelic variant in exon 104 of the fibulin-6 gene (FBLN-6) in some affected individuals with AMD. Other studies, including our own unpublished data, have failed to replicate the presence of this variant in individuals with AMD.

After extensive analysis of the 1q25-q32 region, several groups initially identified a single nucleotide polymorphism (rs1061170) resulting in a T→C change at nucleotide position 1277 of exon 9 of the complement factor H (CFH) gene (MIM 134370) that changes a tyrosine amino acid at position 402 to a histidine (Y402H). The C nucleotide variant at this site has been reported as representing a major risk susceptibility allele for AMD, with all reports indicating between a 2.5- and 7.4-fold increased risk of AMD in the U.S. population.

Follow-up reports have also confirmed these findings. In addition, phenotyping AMD into disease subtypes of either neovascularization, geographic atrophy, or early AMD has indicated that the highest risk associated with the C allele occurs either in early-stage AMD or in neovascular disease. However, the age at diagnosis of AMD did not appear to be altered by this allele.

In the present study, we sought to determine whether the C susceptibility allele of Y402H in the CFH gene was also associated with risk of AMD in the Australian population and whether this risk was dependent on disease phenotype in older patients with AMD.

METHODS

Subjects

All subjects with AMD were identified through either outpatient clinics at the RVEEH or private ophthalmology practices in Melbourne. Control subjects were collected from the same community as part of the large population-based epidemiologic eye study, the Melbourne Visual Impairment Project (VIP) or through aged-care nursing homes.

All individuals (both cases and control subjects) were recruited as part of our AMD inheritance study (AMDIS) and, for inclusion in this study, had to be of Anglo-Celtic ethnic background. In addition, both their parents and all four of their grandparents had to be of Anglo-Celtic origin. All participants were given our standard risk factor and disease history questionnaire, which included questions on the age at diagnosis of AMD, which was taken to be the time the participant

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learned of retinal changes during clinical examination. Thus, the age at diagnosis may have been the time of development of end-stage AMD or, when on routine examination, the patient was noted to have early signs of AMD. The age of ascertainment was the time of enrollment into the AMDIS when a clinical examination was performed, a fundus photograph obtained, and a blood sample collected for DNA analysis. All study participants (cases and control subjects) were examined by a medical retinal ophthalmologist and fundus photographs were taken.

Written informed consent was obtained from all individuals, and ethics approval for the project was provided by the Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital (RVEEH). Melbourne. The study was conducted in accordance with the Declaration of Helsinki and according to the National Health and Medical Research Council of Australia’s statement on ethical conduct in research involving humans, revised in 1999.

The retinal photographs were scored initially for the presence or absence of AMD. Although the strict definition of AMD according to the Wisconsin grading scheme was adhered to in the AMDIS, for a diagnosis of AMD to be made, only those subjects with drusen >125 μm were included in this analysis, to avoid including anyone in the disease group who had marginal clinical signs. Photographs were then graded for the presence of early AMD only (the presence of soft drusen >125 μm, with or without regions of hyperpigmentation) or late AMD (either neovascular or atrophic) being present in at least one eye.

Exclusion criterion were dominantly inherited drusen phenotypes such as Doyne Honeycomb Retinal Dystrophy or malattia leventinese. Individuals with only hard or intermediate drusen (<125 μm) or with only pigmentary changes were not included in the study. Individuals were selected as control subjects based on the presence of a normal fundus (<10 hard drusen <65 μm in size) and no altered macular pigmentation. All AMD cases and control subjects included in the study were unrelated.

Genotyping

Genotyping was performed using a commercial platform (MassArray; Sequenom, San Diego, CA), through the Australian Genome Research Facility, Brisbane, Australia.

In brief, 2.5 ng of genomic DNA was amplified by polymerase chain reaction (PCR) for the T→C substitution at nucleotide position 1277 of exon 9 of the CFH gene, with the primers (forward, 5′-acctgtagttgtattttctgcttacctggactgtaatagtgtaggtggaggattagcttgatc-3′, and reverse, 5′-acggatgcatcggagtagacctctctgctctgctggctgcttgaggtgagccatttcattttggtatgggtcctctaggtgctggt-3′) to give a PCR product of 97 bp. A second validation of results (MassExtend; Sequenom) was initiated by adding DNA polymerase, dNTPs, dNTPs, and an extension primer (5′-ctgtagttgtattttctgcttacctggactgtaatagtgtaggtggaggattagcttgatc-3′), which allowed a 1-bp primer extension of either allelic variant at the polymorphic site. Deoxynucleotides incorporated at the polymorphic site were removed through the use of shrimp alkaline phosphatase (SAP). Two allele-specific products of different masses were synthesized for the T and C alleles through the use of shrimp alkaline phosphatase (SAP).

The PCR consisted of 2 μM MgCl₂, 400 nM of each primer (forward 5′-tttgacaaatgttctgctc-3′, reverse 5′-acggatgcatcggagtagcttgatg-3′) located in the coding sequence of exon 9 of the CFH gene, by using standard concentrations of bovine serum albumin, Taq polymerase, and buffer. An annealing temperature of 55°C was used, and the reaction was run for 25 cycles. For presequencing cleanup, a kit was used (SAP/EXO; USB Corp., Cleveland, OH), and postsequencing cleanup was performed with a spin kit (Dye-Ex 2.0; Qiagen, Valencia, CA). Sequencing was performed with a terminator sequencing kit (BigDye 3.1) on a genetic analyzer (model 3100 with POP6 matrix; Applied Biosystems, Inc., Foster City, CA). A second validation of results was undertaken by repeating the analysis on the gene array platform (MassArray; Sequenom) in 25% of the cases and comparing results between the two runs.

Statistical Analysis

The χ² test was used to calculate the probabilities for the tests of whether there was any (unadjusted) association between various kinds of AMD and different genotypes, and likelihood ratio tests were used for allelic differences. Logistic regression models were constructed to determine the odds ratio (OR) and 95% confidence intervals (CIs) for AMD cases in association with CFH genotypes after adjustment for age and gender. The measurement of attributable risk (AR) for at least one C allele (CC plus CT) and TT genotypes at CFH was calculated with the formula

\[ AR = \frac{(n_0 m_1 - n_1 m_0)}{m_n} \]

where \( n_0 \) and \( n_1 \) denote the frequencies of TT and (CC plus CT) in cases and \( m_0 \) and \( m_1 \) denote the frequencies of TT and (CC plus CT) in control subjects. Independent sample χ² tests were used to calculate the probabilities for the tests of whether there was any difference in mean age of cases and control subjects or mean age at diagnosis of two different genotypes (TT versus CC). Analysis of AMD was undertaken for the subdivision of early, neovascular, and atrophic disease. All analyses were performed in commercial software (SPSS Inc ver. 12.0.1; SPSS Inc, Chicago, IL).

Results

A total of 236 individuals with diagnosed AMD were analyzed in the study (Table 1). In the AMD group, 93 (39.4%) subjects had neovascular disease, 26 (11%) atrophic disease, and 117 (49.6%) early signs of AMD (Table 1). Unrelated but ethnically matched control subjects (mean age, 70 years) were also recruited (Table 1) from Melbourne Overall, the mean age of cases was significantly younger than that of control subjects by 2.9 years (P = 0.04, Table 1) but for end-stage or late disease (neovascular and atrophic), both had an older mean age at

<table>
<thead>
<tr>
<th>Table 1. Characteristics of Study Population: Age and Gender Distribution of AMD Cases and Controls</th>
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<tbody>
<tr>
<td>Early (n = 117)</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Female, n (%)</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
</tr>
<tr>
<td><em>P</em></td>
</tr>
</tbody>
</table>

n (%) is the number of individuals with percentage of the total sample in parenthesis. Probabilities shown are calculated for age of diagnosis versus age control group, with an independent-sample χ² test. The AMD group is stratified based on type of disease.
The genotype and allele frequency distributions for the Y402H single-nucleotide polymorphism (SNP) are shown for cases and control subjects (Table 2) and there was no evidence of departure from Hardy-Weinberg equilibrium for this marker in cases or control subjects. When all individuals with AMD were compared with control subjects, it was evident that the TT or TC genotypes were more frequent in control subjects (9.7%; 46.2% respectively) compared with cases (15.3%; 46.2% respectively; Table 2). In contrast, the CC genotype of all AMD cases was more frequent (38.6%) than in control subjects (9.7%; 46.2% respectively; Table 2). In cases, phenotypic subgroup analysis indicated that the CC genotype was most frequent in individuals with atrophic disease (53.8%) compared with individuals with neovascular disease (40.9%) or those with early signs of disease (35.3%).

Analysis of the alleles at Y402H indicated that the C allele was most frequent in the atrophic group (69.2%) than in the neovascular group (65.1%) and early AMD (57.3%) groups (Table 2). When all AMD cases were compared with control subjects, those with the TC genotype at Y402H had a significantly increased risk of disease (OR 1.86; 95% CI 1.10–3.16; Table 3). This risk dramatically increased in those cases with the CC genotype (OR 9.26; 95% CI 4.52–18.98; Table 3). We therefore subclassified AMD by clinical type and identified a significantly increased risk of AMD of between 2.45- and 4.6-fold and a homozygous C allele resulted in an increased risk of between 3.33- and 7.4-fold.

In this study, the C allele of Y402H in the CFH gene represented a significant risk factor for AMD in our population. Initial studies in U.S. populations reported that the presence of heterozygous CT alleles at this site resulted in an increased risk of AMD of between 2.45- and 4.6-fold and a homozygous C allele resulted in an increased risk of between 3.33- and 7.4-fold. Our study indicated that the presence of heterozygous CT alleles significantly increased the risk of our AMD group by 1.86-fold but in homozygous CC cases, the risk compared with control subjects (Table 3).

### Table 2. Genotype and Allele Distribution at Y402H of the CFH in AMD Cases and Controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Early n (%)</th>
<th>Neovascular n (%)</th>
<th>Atrophic n (%)</th>
<th>Total Case n (%)</th>
<th>Control n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>22 (18.8)</td>
<td>10 (10.8)</td>
<td>4 (15.4)</td>
<td>36 (15.3)</td>
<td>51 (35.4)</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>56 (47.9)</td>
<td>45 (48.4)</td>
<td>8 (30.8)</td>
<td>109 (46.2)</td>
<td>79 (54.9)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>39 (33.3)</td>
<td>38 (40.9)</td>
<td>14 (53.8)</td>
<td>91 (38.6)</td>
<td>14 (9.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3. Odds Ratio (95% CI) of AMD Cases Stratified by Disease Type

<table>
<thead>
<tr>
<th>Genotype</th>
<th>OR (95% CI)*</th>
<th>Total AMD</th>
<th>Early</th>
<th>Neovascular</th>
<th>Atrophic</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>1.86 (1.10–3.16)</td>
<td>1.78 (0.95–3.32)</td>
<td>2.79 (1.21–6.43)</td>
<td>1.01 (0.28–3.67)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>9.26 (4.52–18.98)</td>
<td>6.52 (2.90–14.65)</td>
<td>12.43 (4.61–35.49)</td>
<td>9.61 (2.63–35.09)</td>
<td></td>
</tr>
<tr>
<td>At least 1 C</td>
<td>2.98 (1.81–4.93)</td>
<td>2.54 (1.40–4.61)</td>
<td>4.34 (1.94–9.71)</td>
<td>2.35 (0.74–7.41)</td>
<td></td>
</tr>
<tr>
<td>Female vs. male</td>
<td>2.09 (1.33–3.30)</td>
<td>2.43 (1.39–4.23)</td>
<td>1.86 (0.99–3.51)</td>
<td>1.80 (0.69–4.68)</td>
<td></td>
</tr>
<tr>
<td>Age per year</td>
<td>1.02 (1.00–1.04)</td>
<td>0.98 (0.95–1.00)</td>
<td>1.13 (1.08–1.18)</td>
<td>1.08 (1.02–1.15)</td>
<td></td>
</tr>
</tbody>
</table>

* OR (95% CI), adjusted for age and gender. TT is the reference genotype and the ORs are cases compared with controls.

† Attributable risk defined as at least one C.

### Discussion

In this study, the C allele of Y402H in the CFH gene represented a significant risk factor for AMD in our population. Initial studies in U.S. populations reported that the presence of heterozygous CT alleles at this site resulted in an increased risk of AMD of between 2.45- and 4.6-fold and a homozygous C allele resulted in an increased risk of between 3.33- and 7.4-fold. Our study indicated that the presence of heterozygous CT alleles significantly increased the risk of our AMD group by 1.86-fold but in homozygous CC cases, the risk
increased by 9.26-fold. A more recent paper analyzing a U.S. population also reports a similar OR of 10.05 for homozygote CC individuals. Overall the presence of at least one C allele compared with a T allele increased risk by 2.98-fold in our population. This is slightly higher than the 2.7-fold increased risk previously reported but less than the 5.29 OR also reported in AMD populations.

When AMD cases were analyzed according to subtype, it appeared that the risk associated with the Y402H variant was greater in neovascular AMD than in atrophic disease. However, caution should be exercised with this observation given the relatively small sample size of atrophic individuals in the study. Further recruitment should be undertaken to increase sample size to validate these findings. Of note, the observation of a higher risk of neovascular disease in AMD and variable significance with atrophic disease has been reported before. In that study, it was also reported that association was present with early AMD, and we confirmed a borderline risk in this group.

The population-attributable risk of disease was higher at 57% in the present study compared with previous reports at 43% and at 50% but less than other reports at 68.2%. In the present study compared with previous reports at 68.2%.

However, subtyping of AMD based on clinical disease phenotype indicated that the highest attributable risk resulted from those individuals with neovascular disease, where the attributable risk was 69%.

Of interest, when AMD was analyzed as one group, we did not detect any difference in the mean age at diagnosis of disease in individuals with either a TT or CC genotype (65.2 vs. 65 years; \( P = 0.94 \)). This was in agreement with one study in which no significant difference in age at diagnosis was found in individuals with AMD. However, because age was identified as a significant covariant in our study, we undertook a subgroup analysis. The significantly younger age at diagnosis that we observed in individuals with neovascular disease appeared to reflect dosage of the C allele, whereby individuals with one copy of the C allele presented with a mean age at diagnosis of 4.8 years earlier and those with two copies of this allele had a mean age at diagnosis of 7 years earlier than did individuals with the TT genotype. The atrophic and early AMD groups did not show any difference in mean age at diagnosis with genotype. Of note, in our previous study of the alleles of the apolipoprotein E (apoE) gene, we also found a significant earlier age at diagnosis in only those individuals who presented with neovascular disease and an \( e2/e3 \) genotype.

The risk associated with the Y402H variant suggests a biological role for the C allele, most likely reflecting a functional alteration such as a change in binding property or difference in expression level. It is known that the Y402H variant occurs within I of the 20 short (60 amino acid) consensus repeat sequences that represent important functional domains of the encoded CFH protein. It has been shown that CFH colocalizes with C3b, and both are present in drusen from donor eyes. The CFH protein is a major inhibitor of the alternative complement pathway and thus functions as a defense against infectious agents.

It is of great interest that recently an association between Chlamydia pneumoniae infection and prior cytomegalovirus infection and AMD has been found. Perhaps exposure to these or similar common pathogens in people with a risk-susceptibility allele at Y402H in some way triggers abnormal complement activation that leads to AMD. Confirmation of the CFH gene in AMD by many groups makes the exploration of this and other complement activation scenarios novel areas to research for a better understanding of the risk factors for AMD.

### References


<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sample Size</th>
<th>Age at Diagnosis (Mean ± SD)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>All AMD (( n = 236 ))</td>
<td>TT 36</td>
<td>65.2 ± 15.1</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>CC 91</td>
<td>65.0 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>Early (( n = 117 ))</td>
<td>TT 22</td>
<td>58.0 ± 14.8</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>CC 39</td>
<td>56.7 ± 12.2</td>
<td></td>
</tr>
<tr>
<td>Neovascular (( n = 95 ))</td>
<td>TT 10</td>
<td>78.5 ± 5.9</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>CC 38</td>
<td>71.5 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>Atrophic (( n = 25 ))</td>
<td>TT 4</td>
<td>72.0 ± 1.8</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>CC 13</td>
<td>70.6 ± 6.8</td>
<td></td>
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</table>


**ERRATUM**


In the fourth sentence of the third paragraph of the Methods section, the units for the concentration of 4-(4-(dihexadecylamino)styril)-N-methylpyridinium iodide should be mg/mL.