Variation of Codons 1961 and 2177 of the Stargardt Disease Gene Is Not Associated With Age-Related Macular Degeneration

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Objectives: To investigate the role of 2 specific alleles of the Stargardt disease gene (ABCA4) in the pathogenesis of age-related macular degeneration (AMD). Secondary objectives were to investigate differences in frequency of the G1961E allele in selected ethnic groups as well as to examine the segregation of both G1961E and D2177N alleles in 5 multiplex families with AMD.

Methods: Five hundred forty-four patients with AMD and 689 controls were ascertained from 3 continents. Blood samples from 62 normal individuals of Somalian ancestry were also obtained. Participants were screened for the presence of these ABCA4 alleles with a combination of restriction digestion and single-strand conformation polymorphism analysis of polymerase chain reaction amplification products. Detected alleles were confirmed by DNA sequencing. The number of subjects exhibiting the G1961E or D2177N variants were compared between AMD and control groups using a 2-tailed Fisher exact test.

Results: There was no significant difference (P > 0.1) in the frequency of the G1961E and D2177N alleles in patients with AMD (2.2%) vs controls (1.0%). In contrast, there was a significant difference (P < 0.001) in the frequency of the G1961E alleles between normal individuals of Somalian ancestry (11.3%) and normal individuals from other populations (0.4%). There was no evidence of cosegregation of these alleles and the AMD phenotype in the 5 multiplex families with AMD examined. These two ABCA4 alleles were slightly more frequent in patients with AMD with choroidal neovascularization (2.7%) than those without this complication (2.5%).

Conclusions: Somali ancestry is more than 100 times more strongly associated with presence of the G1961E allele than the AMD phenotype. This study did not find any statistically significant evidence for involvement of the G1961E or D2177N alleles of the ABCA4 gene in AMD.

Clinical Relevance: The ABCA4 gene is definitively involved in the pathogenesis of Stargardt disease and some cases of photoreceptor degeneration. However, it does not seem to be involved in a statistically significant fraction of AMD cases.


A GE-RELATED macular degeneration (AMD) is the most common cause of severe visual loss in the developed world.1 There is a substantial body of evidence that suggests that an important fraction of AMD has a genetic basis.2-6 However, the high prevalence of this disease, its late onset, and its likely mechanistic and genetic heterogeneity have all proved to be significant obstacles to the discovery of the genes involved.

Despite these difficulties, a number of investigators have extensively pursued AMD predisposition genes because of their potential value in the diagnosis and treatment of this devastating condition. As a result, several genes responsible for specific human macular disease phenotypes have been identified.7-13 However, with one possible exception, none of these genes have been found to be associated with a significant fraction of typical late-onset AMD.

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The exception is the ABCA4 gene (formerly ABCR). This gene encodes an adenosine triphosphate–binding cassette transporter of the retina and was convincingly shown by Allikmets et al1 to be responsible for autosomal recessive Stargardt disease. In addition, this gene seems to be capable of causing other retinal phenotypes, including cone dystrophy and retinitis pigmentosa.9,13 Shortly after the original demonstration of this gene’s role in Stargardt disease, Allikmets and colleagues suggested that more than 16% of late-onset macular degeneration was also caused by varia-
PATIENTS, MATERIALS, AND METHODS

PATIENT ASCERTAINMENT

Informed consent was obtained from all study patients or their legal guardians. Five hundred forty-four patients with a clinical diagnosis of AMD were ascertained from the United States (304), Australia (201), and Switzerland (39). Patients were classified as having AMD based on an established international classification system. Clinical information about the patients from the United States and Switzerland was obtained by a retrospective analysis of medical records and fundus photographs, while the Australian patients with AMD were each examined by a small team of ophthalmologists as part of the Australian AMD inheritance study. The average age of patients with AMD ascertained in the United States, Australia, and Switzerland was 75, 78, and 79 years, respectively. All were aged 55 years or older. Of the 235 Iowans with AMD, 110 had definite evidence of choroidal neovascularization (CNV) and 120 were free of this complication. The presence or absence of CNV could not be determined with certainty in the remaining 15. Of the 201 Australian patients with AMD, 99 had definite evidence of CNV, 100 were free of this complication, and 2 had an unknown status. Of the 39 Swiss patients with AMD, 13 had definite CNV and 26 did not. Six hundred eighty-nine control subjects with no history of AMD were ascertained from the United States (408), Australia (187), and Switzerland (94). The minimum age of the control subjects from Switzerland was 25 years; Australia, 51 years; and United States, 40 years.

In addition, blood samples were obtained from 42 unrelated individuals born in Somalia but living in Canada as well as 20 unrelated individuals born in Somalia but living in Australia. All of these individuals had normal vision and had no personal or familial history of any form of macular degeneration, including Stargardt disease.

MOLECULAR ANALYSIS

The DNA was extracted from venous blood as previously described. Exons 42 and 48 (containing codons 1961 and 2177 respectively) of the ABCA4 gene were amplified using previously reported oligonucleotide primers. The G1961E variant was detected using a Taq 1 restriction digestion as follows. The 8.35 µl PCR product was mixed with 1.0 µl 10X Taq 1 buffer, 1 µl 10X bovine specific albumin (BSA), and 4 U of Taq 1 and then incubated at 65°C for 2 hours. Five microliters of the digested product were electrophoresed on 6% polyacrylamide, 5% glycerol non-denaturing gels, and stained with silver nitrate using a standard protocol. The G1961E allele was recognized by the appearance of 2 new fragments 135 base pair (bp) and 77 bp in size and confirmed by automated DNA sequencing using dye-terminator chemistry and an ABI 377 sequencer. The D2177N allele was detected by single-strand conformation polymorphism analysis. Amplifiers were denatured at 93°C for 3 minutes, electrophoresed on 6% polyacrylamide, 3% glycerol non-denaturing gels, and stained with silver nitrate as mentioned earlier in this article. Samples exhibiting band shifts were sequenced as described earlier. All samples were screened in a single laboratory, and all gels were examined by a minimum of 2 experienced investigators. In addition, the samples from the Australian patients were independently screened in a second laboratory with identical results.

STATISTICAL ANALYSIS

The proportion of subjects showing the G1961E or D2177N variant were compared between AMD and control groups using the Fisher exact test (2-tailed). This analysis was calculated for the sample group as a whole and for each individual subgroup. Correction for multiple measurements and the power calculation were performed as described elsewhere.

RESULTS

The distributions of G1961E and D2177N sequence variations in the various AMD and control populations are summarized in Table 1. Overall, 544 patients with AMD and 689 ethnically matched population controls were studied. Five instances of the G1961E variation and 7 instances of the D2177N change were observed among the patients with AMD, while 3 instances of G1961E and 4 of D2177N were observed among the controls. These changes were heterozygous in all 12 cases. A Fisher exact test revealed the differences in allele frequency between these groups to be insignificant whether the alleles were considered together or separately (P>0.10 in all cases) even without correction for multiple measurements. Collectively, the G1961E and D2177N sequence changes were found in 2.2% of the 544 patients with AMD in this study, ranging from 0.99% in patients from the United States to 5.1% in patients from Switzerland. This variation suggested that ethnic differences in ABCA4 allele frequencies might exist to a degree that could
affect the interpretation of a study if they were not taken into account.

The latter idea was strengthened by a chance observation made during our study of a large group of probands with Stargardt disease (Andrew R. Webster, MD, FRCOphth, E.H., A.J.L., et al, unpublished data, February 2000). Specifically, Webster and co-workers found that only 5 of 386 Stargardt probands were homozygous for a likely disease-causing mutation in the ABCA4 gene. Two of these 5 individuals were homozygous for G1961E, and both of them were patients with Somali ancestry. The fact that these were the only 2 Stargardt probands in the entire cohort who were known to have Somali ancestry suggested that the G1961E allele frequency might be much higher in individuals from Somalia than those from other ethnic backgrounds. To test this hypothesis, 62 unrelated normal individuals with Somali ancestry were screened for the G1961E change, and 7 were found to be heterozygous for this variant (11.2%).

Table 2 gives the result of sequential comparisons (Fisher exact test) of the frequency of the G1961E change in normal individuals from Somalia and normal individuals from our other study populations. The frequency of the G1961E change is significantly greater in unaffected individuals from Somalia than in unaffected individuals from other ethnic backgrounds. Table 3 gives a similar set of sequential comparisons between the frequency of the G1961E change in normal individuals from Somalia and the frequency of the change in patients with AMD in our study. The frequency of the G1961E change is significantly greater in unaffected individuals from Somalia than in the entire cohort of patients with AMD in this study (P < .001).

Five of the 7 AMD probands from Australia who harbored a G1961E or D2177N change had family members who also carried the clinical diagnosis of AMD. Clinicians who were masked to the molecular status of these individuals ascertained all living siblings of the affected individuals and diagnosed them as affected (Figure 1A) or unaffected (Figure 1B) with AMD. Figure 2 shows the results of these examinations as well as the associated molecular findings. Of the 15 family members diagnosed with AMD, 8 harbored a G1961E or D2177N change. Similarly, of the 5 siblings who were clinically unaffected, 3 had the ABCA4 sequence variation that was present in the proband of their family.

Table 1. G1961E Association With Age-Related Macular Degeneration

<table>
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<tr>
<th>Location</th>
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<tr>
<td>with AMD</td>
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<tr>
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</tr>
<tr>
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<td>.34</td>
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</tr>
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* AMD indicates age-related macular degeneration; ellipses, not applicable.

Table 2. G1961E Association With Ethnicity (Somali Controls vs Other Controls)*

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<td>Switzerland</td>
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<td>3</td>
<td>&lt;.001</td>
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* Ellipses indicate not applicable.

Table 3. G1961E Association With Ethnicity (Somali Controls vs Patients With AMD)*

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<th>Location</th>
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<td>Iowa</td>
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<tr>
<td>Somalia</td>
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</table>

*AMD indicates age-related macular degeneration; ellipses, not applicable.

Perhaps the greatest challenge in interpreting the clinical significance of sequence variations in the ABCA4 gene...
et al initially examined a cohort of patients with Stargardt disease, patients with AMD, and controls, they found more than 1100 instances of 140 different sequence variations. In principle, any of these sequence variations could be disease causing while in fact, many of these changes probably represent non–disease-causing polymorphisms. Before screening a population of patients for potential disease-causing variations in a gene, it is difficult to know what types of variations are likely to be found. However, if one uses the distribution of different alleles between patients and controls as the criterion for determining the pathogenicity of those alleles, it is statistically invalid to use the same set of data to argue that the gene is involved in the pathogenesis the disease (ie, based on the skewed distribution of “pathogenic” alleles). It would be valid to use the first set of data as a pilot experiment to identify the characteristics of likely disease-causing mutations and then to sample a new population to see whether the hypothesis generated from the first experiment can be supported by the second. Therefore, in this study, we focused on the 2 sequence variations (G1961E and D2177N) that were most plausibly associated with AMD in the study by Allikmets et al. In that study, Allikmets and colleagues found evidence that one or the other of those 2 sequence variations were found in approximately 8% of patients with AMD (one-half of the total ABCA4 association they observed). Although these 2 sequence variations were statistically associated with AMD when considered by themselves, there was no statistical association with AMD if all of the observed missense variations were included in the analysis, or alternatively, if all of the various subgroup analyses were subjected to a statistical correction for multiple measurements. In this study, we failed to find any statistically significant association between AMD and the presence of the G1961E and D2177N ABCA4 sequence variations. We found these 2 ABCA4 variations in 12 (2.2%) of 544 patients with AMD. This was similar to the frequency we observed in patients with AMD in our previous study (3 [1.6%] of 182) but more than 3-fold lower than that observed by Allikmets and coworkers (13 [7.8%] of 167). With the sample size that we used, our study had a power of greater than 95% (α = .05) to detect the difference in frequency of these alleles between patients with AMD and controls who had been previously reported (7.8% vs 0.5%). However, it should be emphasized that the control individuals in the present study were not examined for the presence of AMD and that many of them were not old enough to manifest the signs of the disease even if they had been examined. As a result, a portion of these control subjects would be expected to harbor the same AMD-causing variations that were present in an AMD patient cohort (thereby lessening the difference between the 2 groups). If one assumes that one third of the population is at risk for the degree of AMD manifested by the patients in this study, one would predict a 3-fold greater

Figure 1. A, Fundus photograph of the right eye of a 71-year-old woman (family 1, patient 3 in Figure 2) with age-related macular degeneration (AMD). Her visual acuity was 20/200 OD. Although 5 of her 6 siblings harbored the G1961E allele, she did not. B, Fundus photograph of the left eye of a 66-year-old woman (family 1, patient 5 in Figure 2). Although this woman did harbor the G1961E allele, the clinicians who examined her felt that she was unaffected by AMD.

Figure 2. Pedigree drawings of 5 Australian families with age-related macular degeneration (AMD) and ABCA4 sequence variations. Closed symbols indicate the presence of a heterozygous ABCA4 sequence variation that was present in the family’s proband (G1961E for family 1, and D2177N for families 2-5). Fundus photographs of 2 members of family 1 are shown in Figure 1, while photographs of 4 members of family 2 are shown in Figure 3. The age (y) of each individual is shown beneath their pedigree symbol, while the proband of each family is identified with an arrow.
frequency of a true disease-causing mutation in the AMD cohort than in an unexamined and/or young control population. The sample size of this study was also large enough to result in a 95% power (\(\alpha = .05\)) to detect such a 3-fold difference if the frequency in the AMD cohort was as large as had been previously suggested (ie, 7.8% vs 2.6%).

An interesting observation in the study by Allikmets and coauthors was that the G1961E and D2177N sequence variations were more commonly found in patients who had not experienced CNV. Specifically, only 1 of their 33 patients with ABCA4 sequence variations had experienced that complication. In the present study, we found no evidence to support the idea that ABCA4 sequence variations are more likely to be present in patients with AMD who do not have CNV. Of the 12 patients with AMD in this study who harbor G1961E or D2177N sequence variations, 6 had CNV.

One of the difficulties of studying disease-associated allelic variation in ethnically diverse human populations is that some of the variation that is observed may be secondary to unrecognized variations in ethnicity instead of variations in the disease state. High frequencies of certain alleles can be present in certain ethnic groups because of geographic or cultural barriers that existed hundreds of years earlier. It often requires a chance observation to discover a population in which a specific allele is enriched or depleted because the number of different ancestral ethnic groups worldwide is so great. For example, in this study, we found that the frequency of the G1961E allele was significantly higher in patients of Somali ancestry than in control populations from the United States, Australia, and Switzerland (Table 2) or AMD populations in the United States and Australia (Table 3). Although further work needs to be performed to carefully characterize the phenotype of G1961E heterozygotes with Somali ancestry, the point to be made in the present context is that Somali ancestry is more than 100 times more highly correlated to the presence of the G1961E sequence variation than the presence of AMD.

A common strategy for inferring the pathogenicity of sequence variations in human populations is to demonstrate a cosegregation of the variation and the phenotype in a large number of individuals. This is the basis of the familiar lod score method for chromosomal localization of disease genes. Some authors have sought to investigate the possible role of ABCA4 sequence variations in macular degeneration by examining the cosegregation of the AMD phenotype with certain ABCA4 alleles in patients with familial AMD. These studies have taken 2 forms. In the first, the grandparents of patients with Stargardt disease are studied to determine whether the 2 grandparents who harbor one of the ABCA4 alleles present in the affected child also manifest AMD. In the second, siblings of an AMD proband with a variant ABCA4 allele are examined for the cosegregation of the AMD phenotype and the ABCA4 allele. For this type of experiment to be valid, it is necessary for the clinicians making the phenotypic judgments to be masked to

Figure 3. A, Fundus photograph of the right eye of an 81-year-old woman (family 2, patient 1 in Figure 2) with age-related macular degeneration (AMD) and choroidal neovascularization (CNV). Her visual acuity in this eye was hand motions. She was found to harbor the D2177N allele. B, Fundus photograph of the left eye of an 80-year-old woman (family 2, patient 2 in Figure 2) with AMD and CNV. Her visual acuity in this eye was hand motions. She was found to harbor the D2177N allele. C, Fundus photograph of the left eye of a 66-year-old man (family 2, patient 3 in Figure 2) with AMD and CNV. His visual acuity was 20/100 OS. Although 2 of his 3 relatives harbored the D2177N allele, he did not. D, Fundus photograph of the right eye of a 78-year-old woman (family 2, patient 4 in Figure 2) with AMD. Her visual acuity was 20/100 OD. Although 2 of her 3 relatives harbored the D2177N allele, she did not.
the genotypic information and for all of the relatives of the proband to be equally ascertained. In addition, an adequate number of individuals need to be examined to allow the possibility of observing a statistically significant association. None of the studies currently in the literature have demonstrated a statistically significant association of ABCA4 alleles with an AMD phenotype in this type of experiment.

In the present study, we examined all of the siblings of 5 families affected with AMD who were also found to harbor a G1961E or D2177N ABCA4 allele. In all cases, we observed at least one individual with the AMD phenotype who did not harbor the proband’s ABCA4 allele, and in 2 of the families, there were also unaffected siblings who did harbor the proband’s allele (Figure 2). These findings by themselves do not demonstrate that ABCA4 alleles lack a phenotype in the heterozygous state. That is, if these alleles represent only a small fraction of all patients with AMD, one would expect to find many examples in which some other genetic cause of AMD was present within a nuclear family. Similarly, the absence of the phenotype in individuals who carry a certain allele can be explained by the incomplete penetrance that is a common feature of all late-onset dominant diseases. However, these 5 families do illustrate the difficulty that one encounters trying to establish a statistically significant association between certain ABCA4 alleles and a common phenotype like AMD.

The data in this article notwithstanding, there is no doubt that variations in the ABCA4 gene cause human retinal disease. The data supporting the involvement of this gene in autosomal recessive Stargardt disease are extensive, and the data for its role in other recessive retinal degenerations are also strong. However, much remains to be learned about the phenotypic effect of specific alleles. The fact that 11% of people of Somali ancestry are heterozygous for the G1961E allele would predict that more than 1% of that population would be homozygous. The fact that Stargardt disease is not known to be 100 times more prevalent in this population than in the United States, Switzerland, or Australia suggests that G1961E does not frequently cause disease in the homozygous state. It raises the testable hypothesis that G1961E is more likely to cause disease in the compound heterozygous state than in the homozygous state. Thus at the present time, it seems that we do not fully understand the pathogenic role of the G1961E allele even in patients with Stargardt disease (with which the allele has been significantly associated in multiple studies). It therefore seems premature to suggest a pathogenic role for this allele in a much more common and more genetically heterogeneous disease (AMD).

Given the extreme allelic diversity of the ABCA4 gene in the human population, the most straightforward way to investigate the possibility of its role in dominantly inherited (ie, heterozygous) late-onset disease may be with the use of transgenic animal models and/or in vitro systems.

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REFERENCES


