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Report

Further Evidence for an Association of *ABCR* Alleles with Age-Related Macular Degeneration

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Age-related macular degeneration (AMD) accounts for >50% of the registered visual disability among North American and Western European populations and has been associated both with environmental factors, such as smoking, and with genetic factors. Previously we have reported disease-associated variants in the *ABCR* (also called *ABCA4*) gene in a subset of patients affected with this complex disorder. We have now tested our original hypothesis, that *ABCR* is a dominant susceptibility locus for AMD, by screening 1,218 unrelated AMD patients of North American and Western European origin and 1,258 comparison individuals from 15 centers in North America and Europe for the two most frequent AMD-associated variants found in *ABCR*. These two sequence changes, G1961E and D2177N, were found in one allele of *ABCR* in 40 patients (~3.4%), and in 13 control subjects (~0.95%). Fisher's two-sided exact test confirmed that these two variants are associated with AMD at a statistically significant level ($P < .0001$). The risk of AMD is elevated approximately threefold in D2177N carriers and approximately fivefold in G1961E carriers. The identification of a gene that confers risk of AMD is an important step in unraveling this complex disorder.

Age-related macular degeneration (AMD, also designated "ARMD2" [MIM 153800]) accounts for >50% of the registered visual disability among the North American and Western European populations and has been associated with both environmental and genetic factors (Klein et al. 1992; Vingerling et al. 1995). Previously we reported disease-associated variants in the Stargardt disease gene, *ABCR* (Allikmets et al. 1997*b*), in a subset of patients affected with this complex disease trait (Allikmets et al. 1997*a*). Challenges (Dryja et al. 1998) to this report have included the following: (1) controls were not screened as intensively as were subjects with AMD, (2) statistical corrections were not made for multiple comparisons, and (3) the association might be due to inadequate "racial matching"—that is, population stratification. Also, one investigation could not find an association between *ABCR* alterations and AMD (Stone et al. 1998).

We present new data on 1,218 patients with AMD and 1,258 matched controls studied at 15 centers (7 in the United States and 8 in Europe) to test the associations with AMD of two of the more common AMD-associated *ABCR* variants, G1961E and D2177N. Because all case and control subjects were tested in a coded (masked) fashion at each center and because this is an independent confirmatory study, the design responds to objections (1) and (2) above. Conducting the study in 15 centers in the United States and Europe minimizes the possibility of population stratification (selection bias). Each center in this consortium recruited patients with AMD defined according to published criteria (Bird et al. 1995; Age-Related Eye Disease Study Research Group 1999), and patients were categorized as having either dry (drusen and retinal pigmentary epithelial abnormalities and/or geographic atrophy) or wet (exudative or neovascular) forms of AMD by clinicians without prior knowledge of the molecular genetic analyses. The mean age (± SD) of patients from all centers was 74.8 ± 7.4 years. Controls were matched to case subjects by race and age in some centers, whereas general population (GP) racially matched controls were used in other centers (table 1).

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Table 1

Association of G1961E and D2177N Alleles with AMD

		AMD Samples			Controls			G1961E		D2177N	
Center	Population	Dry	Wet	Total	GP	AM	Total	AMD (D/W)	Control (GP/AM)	AMD (D/W)	Control (GP/AM)
Boston/Salt Lake City/Baltimore	Eur. Am.	185	44	229		200	200	3 (3/0)	0	6 (6/0)	1 (0/1)
Houston	Eur. Am.	55	46	101		100	100	1 (1/0)	0	1 (0/1)	0
Los Angeles	Eur. Am.	53	50	103	158		158	5 (3/2)	1 (1/0)	4 (2/2)	0
Wuerzburg	German	100	100	200		100	100	4 (1/3)	1 (0/1)	3 (2/1)	2 (0/2)
Nijmegen	Dutch	24	59	83	168		168	1 (0/1)	0	1 (1/0)	3 (3/0)
Rotterdam/Amsterdam	Dutch	36	79	115		100	100	0	1 (0/1)	0	1 (0/1)
Naples/Milan	Italian	31	61	92	120	51	171	3 (2/1)	0	0	0
Barcelona	Spanish	10	26	36		34	34	0	0	1 (0/1)	0
Uppsala	Swedish	20	82	102	100		100	1 (1/0)	1 (1/0)	2 (1/1)	0
London	British	15	75	90		20	20	1 (0/1)	0	1 (0/1)	0
Paris	French	4	63	67	107		107	0	0	2 (0/2)	1 (1/0)
Total (%)		533	685	1,218	653	605	1,258	19/1,218 (1.56)	4/1,258 (.32)	21/1,189 (1.77)	8/1,258 (.64)
P								*P* = .0013		*P* = .014	
OR (95% CI)								5.0 (1.6–20)		2.8 (1.2–7.4)	
Both variants								40/1,189 (3.36%)	12/1,258 (.95%)	*P* < .0001	

Note.—AM = age-matched; D = dry form of AMD; W = wet form of AMD; SLC = Salt Lake City; Eur. Am. = European American; OR = odds ratio. *P* values were calculated from the two-sided Fisher's exact test, and ORs were calculated from the exact conditional hypergeometric distribution (Mehta and Patel 1995).

The mean ages of controls were 73.4 ± 6.6 years for the age-matched subset and 39.2 ± 8.2 years for the GP sample. Each age-matched control received a complete eye examination, to exclude AMD. Eye examinations were not performed on all GP controls, since these individuals were relatively young (39.2 ± 8.2 years) and, therefore, not at risk for AMD.

Genotyping of the G1961E and D2177N variants was performed by a method of choice at each center. These methods, all described elsewhere, included PCR-RFLP scanning (Deng 1988), SSCP analysis (Orita et al. 1989; Allikmets et al. 1997*b*), denaturing gradient gel electrophoresis (Myers et al. 1985), allele-specific oligonucleotide hybridization (Conner et al. 1983), and amplification refractory mutations system allele-specific PCR hybridization (Newton et al. 1989). Positive controls for the two variants were provided to each center, to ensure a 100% detection rate and to eliminate false-negative results. All positive cases were confirmed by direct sequencing. Data from the 15 centers were aggregated into 11 analysis units by grouping centers that collaborated closely (table 1).

G1961E was found in 19/1,218 (1.56%) patients with AMD compared with 4/1,258 (0.32%) control subjects (table 1) (Fisher's two-sided exact test [Mehta and Patel 1995]; *P* = .0013). For the pooled data, the odds ratio, calculated from the exact conditional hypergeometric distribution (Mehta and Patel 1995), is 5.0 (95% confidence interval [CI] 1.6–20]). An exact conditional analysis stratified on analysis unit yielded an odds ratio of 5.2 (95% CI 1.7–22). No statistically significant evidence of heterogeneity of odds ratios exists among analysis units, and the prevalence is higher in the AMD group in six of the eight units with at least one G1961E allele.

D2177N was present in 21/1,189 (1.77%) AMD cases, compared with 8/1,258 (0.64%) controls (*P* = .014). The pooled odds ratio, 2.8 (95% CI 1.2–7.4) was similar to the odds ratio estimated by stratification on each unit, 2.6 (95% CI 1.1–7.0). There was no statistically significant evidence of heterogeneity of odds ratios among units, and the prevalence of D2177N was higher in AMD cases in 8 of the 10 units with at least one D2177N allele.

The combination of either G1961E or D2177N was found in 40/1,189 (3.36%) patients with AMD, compared with 12/1,258 (0.95%) control subjects (*P* < .0001). The study design was directed toward increasing the power of statistical analysis by increasing the sample size substantially with the pooling of data from all study centers. However, if analyzed separately or in small subsets, the data from almost all study centers do not show statistical significance. This outcome is not surprising when one views the observed low allele frequency of these two variants. For example, analysis of the data previously reported by Stone et al. (1998) for these two variants (3/182 in patients, 0/96 in control subjects) by itself suggests no association. However, in the context of the current study, these data (1) correlate perfectly with the results from all study centers, and (2) if included, increase the statistical significance of the association.

We have reported elsewhere that *ABCR* variants are more prevalent among subjects manifesting the dry (nonneovascular) form of the disease (Allikmets et al. 1997*a*). Distinction between the dry and wet phenotypes is not unambiguous (Stone et al. 1998). Whether or not these two clinical manifestations have different genetic risk factors remains to be elucidated. Occasionally, individuals with the dry form in one eye may develop choroidal neovascularization later in life in either the same or the other eye (Sunness et al. 1999). Each participating clinician in this consortium segregated its patient population by phenotype, between those with the dry form, characterized by drusen, retinal pigment epithelium (RPE) pigmentary abnormalities, and/or geographic atrophy; and those manifesting the wet, or exudative (neovascular), stage (Bird et al. 1995; Age-Related Eye Disease Study Research Group 1999). For the purposes of these analyses, when different phenotypes appeared in the two eyes, the assignment of the disease status was based on the more severely affected eye.

Of the 1,218 patients analyzed, 533 had the dry phenotype and 685 had the exudative form (table 1). The proportion of patients diagnosed with the exudative complication was higher in European centers than in North American centers (table 1). Together, the G1961E and D2177N variants were present in 23/533 patients with nonexudative disease and in 17/685 with exudative lesions. The difference is marginally significant (P = .027) with the one-sided Fisher's exact test (Mehta and Patel 1995). The pooled odds ratio was 1.77 (95% CI 0.9–3.4). Although we have demonstrated that variants in *ABCR* may be associated predominantly with nonexudative AMD, the clinical relevance of this finding remains to be determined. Future studies, with larger numbers of affected individuals and control subjects, may be able to link allelic variants in *ABCR* to specific disease phenotypes.

The results remain statistically significant when the data are stratified by the type of control—age-matched or GP. The combination of either variant occurs in 24/863 (2.8%) cases and in 6/605 (0.99%) age-matched controls, a statistically significant difference (P = .008 with one-sided Fisher's exact test). Of interest, the association is stronger with GP controls—16/355 (4.5%) in patients, 6/653 (0.92%) in control subjects (P = .0003). This could be explained, however, with a relatively small patient sample size (355) applicable for the analysis with the GP controls. Furthermore, we cannot exclude that some of the heterozygous GP control subjects could develop AMD later in life, since their mean age is well below 60 years.

For population stratification to explain the above associations at any one study center, ethnic (sub)groups in that center should both associate strongly with AMD prevalence and segregate concordantly with the prevalences of both G1961E and D2177N. We know no data that suggest substantial variation of AMD prevalence across ethnic subgroups within the white population; indeed, the Chesapeake Bay Watermen Study and the Beaver Dam Eye Study in the United States and the Rotterdam Eye Study yield similar age-specific prevalences of AMD (see fig. 1 in Vingerling et al. 1995). Moreover, the direction of the association is the same in European centers, within which populations are relatively homogeneous, as in US centers, within which ethnic diversity is greater. Although the US data yield larger estimated odds ratios than do the European data, formal tests for differences between these odds ratios are not statistically significant. It also seems unlikely that similar ethnic correlations should occur with G1961E and D2177N, which we have yet to find together in the same subject. Finally, even if population stratification accounted for an association in one analysis unit, it is difficult to imagine that it could possibly account for associations in so many diverse, geographically distributed populations (table 1).

Other lines of evidence suggest a possible role for *ABCR* in AMD. Several reports have indicated a higher rate of AMD in parents and grandparents of patients with recessive Stargardt disease harboring *ABCR* variants (Rozet et al. 1998; Lewis et al. 1999; Shroyer et al. 1999; Souied et al. 1999). Data from photoaffinity labeling and ATPase activity experiments (H. Sun, P. M. Smallwood, and J. Nathans, personal communication) indicate that both variants affect the protein function in vitro. The mutant G1961E protein, produced after the transfection of human embryonic kidney (293) cells with cloned cDNA, exhibits several-fold lower binding of 8-azido-ATP and *inhibition* of ATPase activity by retinal, as compared with the wild-type ABCR protein. The D2177N variant had no effect on 8-azido-ATP binding but exhibited a reproducible *elevation* in ATPase activity relative to the wild type (H. Sun, P. M. Smallwood, and J. Nathans, personal communication). The physiological consequences of the latter observation have to be further elucidated. Functional studies of ABCR have implicated all-*trans* retinal or its conjugates with phosphatidylethanolamine (PE), N-retinylidene-PE, and N-retinylidene-N-retinylethanolamine (A2E), as potential substrates for this transporter protein (Sun et al. 1999; Weng et al. 1999). Mice homozygous for a null mutation in *ABCR* accumulate high levels of a major fluorophore of lipofuscin, A2E, in the RPE, a phenotype associated with AMD in humans (Weng et al. 1999). Most recently, A2E accumulation was also demonstrated in the RPE of $Abcr^{+/-}$ heterozygote mice, but at a slower, age-related rate (Mata et al. 2000). The distinct AMD-resembling phenotype in $Abcr^{+/-}$ mice suggests that humans heterozygous for *ABCR* mutations may be predisposed to

A2E accumulation and concomitant retinal or macular disease (Mata et al. 2000).

In summary, new data from 15 centers in the United States and Europe independently confirm the association of *ABCR* alleles G1961E and D2177N with AMD. Studying diverse populations minimizes the chance that the associations are due to population stratification. That similar masked assays were performed on both case and control subjects and that the new confirmatory data achieved statistical significance should allay earlier concerns about unequal thoroughness of assays and multiple comparisons (Dryja et al. 1998). We welcome other studies, such as sib-based transmission/disequilibrium tests, to test against the possibility of population stratification, but such designs have their own weaknesses and would require much larger populations and more-difficult sample collection than did the present case-control study (Schaid and Rowland 1998). Our experience indicates that successful association analysis can be accomplished by formation of large international consortia with carefully planned and unified protocols.

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