Genotype–Phenotype Analysis of *ABCR* Variants in Macular Degeneration Probands and Siblings

Paul S. Bernstein,[1] *Mark Leppert,*[2] *Nanda Singh,*[2] *Michael Dean,*[3] *Richard Alan Lewis,*[4,5,6,7,8] *James R. Lupski,*[5,7] *Rando Allikmets,*[9,10] *and Johanna M. Seddon*[11]

Purpose. Single-copy variants of the autosomal recessive Stargardt disease (STGD1) gene *ABCR* (*ABCA4*) have been shown to confer enhanced susceptibility to age-related macular degeneration (AMD). To investigate the role of *ABCR* alleles in AMD further, genotype–phenotype analysis was performed on siblings of patients with AMD who had known *ABCR* variants. This genetically related population provides a cohort of subjects with similar age and ethnic background for genotype–phenotype comparison to the original probands.

Methods. All available siblings of 26 probands carrying probable disease-associated *ABCR* variants were examined clinically. Blood samples were collected from these siblings for genotype analysis to search for the *ABCR* variant alleles corresponding to the isofamilial proband.

Results. Nineteen of 33 siblings from 15 families carried the respective proband's variant *ABCR* allele. Some families exhibited concordance of *ABCR* alleles with macular degeneration phenotype, but others did not. Exudative AMD was uncommon among both probands and siblings.

Conclusions. Although population studies have indicated that some *ABCR* variant alleles may enhance susceptibility to AMD, investigation of the extent of *ABCR* involvement by kindred analysis is complicated by a plethora of environmental and other hereditary factors not investigated in the current study that may also play important roles. (*Invest Ophthalmol Vis Sci.* 2002;43:466–473)

The *ABCR* (*ABCA4*) gene codes for a retina-specific protein on the rims of photoreceptor outer segment discs that appears to facilitate the transport of retinoids during the visual cycle.[1,2] Homozygous and compound heterozygous mutations in *ABCR* are responsible for recessive Stargardt macular dystrophy (STGD1),[3–10] some cases of recessive retinitis pigmentosa,[11] and a substantial fraction of autosomal recessive cone-rod dystrophies.[12,13] Because STGD1 shares phenotypic similarities with age-related maculopathy (ARM) and age-related macular degeneration (AMD), we have screened previously for *ABCR* sequence variants in clinic populations with moderate to advanced nonexudative and disciform AMD. We reported that 16% of an initial cadre of patients with AMD (26/167) had heterozygous *ABCR* variants that resulted in nonconservative amino acid substitutions, frameshifts, or splice-site changes that were found in less than 1% of a general population control cohort.[14] Two variants, G1961E and D2177N, accounted for half of the reported disease-associated variants, whereas the others were rare variants found in one or two affected individuals.[14]

Two groups subsequently reported much lower rates of potential disease-associated *ABCR* variants in their cohorts of patients with AMD,[15–17] but their selected populations, clinical criteria, and mutation detection rates differed substantially from the initial study.[18] More recently, however, a large multicenter international consortium confirmed that G1961E and D2177N variants of *ABCR* are indeed found in patients with AMD at a significantly higher frequency relative to control subjects.[19] The two variants were found in 3.4% of patients with AMD (40/1189) versus 0.95% of control subjects (12/1258; $P < 0.0001$).[19]

We postulate that relatives of patients with Stargardt disease and of patients with AMD who are heterozygous carriers of the same variant *ABCR* alleles as the family proband may have an increased risk of development of AMD under some circumstances. Several groups have reported pedigrees in which *ABCR* variants cosegregate with AMD in parents and grandparents of STGD probands,[20–22] but the number of available families with two or three surviving generations has been limited. In this study we examined and genotyped all available siblings of the subjects with disease-associated *ABCR* variants from our initial study of *ABCR* in AMD,[14] and we obtained additional information on the phenotype of the family probands. Our goal was to determine the concordance of mutant *ABCR* alleles with the AMD phenotype in these families.

Materials and Methods

All subjects found to have AMD-associated *ABCR* variants from the initial study of *ABCR* in AMD[14] were asked to provide contact information for all living siblings. Available siblings were examined by one of the authors (PSB or JMS) or by the subject's personal ophthalmologist if the patient was unable to visit either Salt Lake City or Boston.

From the [1]Department of Ophthalmology and Visual Sciences, Moran Eye Center, University of Utah School of Medicine, Salt Lake City, Utah; the [2]Department of Human Genetics, University of Utah, Salt Lake City, Utah; the [3]Laboratory of Genomic Diversity, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland; the Departments of [4]Ophthalmology, [5]Pediatrics, [6]Medicine, [7]Molecular and Human Genetics, and the [8]Huffington Center on Aging, Baylor College of Medicine, Houston, Texas; the Departments of [9]Ophthalmology and [10]Pathology, Columbia University, New York, New York; and the [11]Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts.

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Corresponding author: Paul S. Bernstein, Department of Ophthalmology and Visual Sciences, Moran Eye Center, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, UT 84132; paul.bernstein@hsc.utah.edu.

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TABLE 1. AMD Diagnostic Criteria

Grade 1: no drusen or nonextensive small (hard) drusen, without pigment abnormalities
Grade 2: extensive small (hard) drusen or pigment abnormalities
2a: drusen
2b: RPE changes (hypo- or hyperpigmentation)
2c: both
Grade 3: extensive intermediate drusen or any large drusen
3a: no drusenoid RPE detachment
3b: drusenoid RPE detachment
Grade 4: geographic atrophy
Grade 5: exudative AMD
5a: serous RPE detachment
5b: CNVM or disciform scar

Drusen size: Small, <63 μm; intermediate, 63–125 μm; large >125 μm. CNVM, choroidal neovascular membrane.

Fundus photographs were then taken, and blood samples were obtained for genotyping. This program complied with the policies of the institutional review boards of the participating institutions and with the Declaration of Helsinki, and all subjects supplied provided informed consent.

Grading of fundus photographs was with the five-level scale for AMD classification used in previous studies (Table 1).[23,24] It is a modification of the scale used in the Age-Related Eye Disease Study (AREDS).[25] Fundus photographs were graded independently by the two clinical graders (PSB and JMS) to assure consistency. Graders were unaware of the subjects' clinical histories if the subjects did not originate from his or her site. Only rarely were the scores of the graders discordant, and in these cases (<5% of photographs), the two graders reviewed these photographs together and achieved a consensus grade. When an individual's right and left eyes had different grades, the higher grade was used for data analysis.

Sibling genotyping for the proband's *ABCR* mutation was performed on samples of peripheral blood by direct DNA sequencing with either of two dye terminators (ABI dRhodamine or Prism BigDye; PE Applied Biosystems, Foster City, CA) and cycle sequencing with DNA polymerase (*Taq* FS; PE Applied Biosystems). DNA sequence was analyzed on an automated DNA sequencer (ABI Prism 377) according to the manufacturer's protocols (PE Applied Biosystems). The χ^2 statistic with the Cochran-Mantel-Haenszel option obtained odds ratios and 95% confidence intervals.

RESULTS

Genotype-Phenotype Characterization of the Original Cohort of Patients with AMD

Family probands carrying probable disease-associated *ABCR* variants had been identified in our earlier report of the role of *ABCR* in AMD.[14] Before studying *ABCR* variants in their siblings, we characterized the initial cohort of subjects in a systematic manner for trends in genotype–phenotype correlations that could be investigated further in these siblings. Fundus photographs from the 167 participants in the initial study were graded according to the system outlined as Table 1. The clinical phenotypes observed for specific *ABCR* alleles in the patients with AMD with disease-associated *ABCR* variants are in Table 2. Of the participants, 96.4% had moderate to advanced AMD (grades 3–5), whereas only 3.6% had early (borderline) age-related maculopathy (grade 2; Table 3). No subjects were unaffected (grade 1). Analysis of these data confirmed that possession of an *ABCR* variant was associated significantly with a dry nonexudative phenotype in subjects with moderate to advanced AMD, because the 95% confidence intervals for grades 3 and 4 compared with grade 5, although broad, did not overlap with 1.0. This result is important, because other studies in which investigators failed to find an association between *ABCR* variants and AMD enrolled much higher numbers of subjects with exudative (grade 5) AMD (>60% versus 26% in our initial study).[15,16]

Further comparison of the initial study participants revealed the following characteristics, shown in Table 4: Subjects with *ABCR* variants had slightly earlier ages of AMD diagnosis relative to *ABCR*-negative subjects, they were slightly more likely to be male, and they were more likely to have a self-reported family history of AMD, although these differences were not statistically significant. Subjects with *ABCR* variants were more likely to have identical AMD grades in both eyes relative to *ABCR*-negative subjects—a result with borderline statistical significance, because the lower end of the 95% confidence interval was 1.0.

Genotype-Phenotype Characterization of Siblings of Subjects with AMD Variants

If an AMD family proband carries a heterozygous *ABCR* variant allele, each of his or her siblings has a 50% chance of carrying the same *ABCR* variant. Because typically they are close in age

TABLE 2. AMD Grade of Probands Carrying Heterozygous *ABCR* Variants

ABCR Variant	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
E471K	0	0	1	1	0
P940R*	0	0	0	1	0
T1428M	0	0	1	0	0
R1517S	0	0	0	1	0
I1562T	0	0	1	1	0
G1578R	0	0	1	0	0
5196+1G→A	0	0	1	0	0
R1898H	0	0	0	1	0
G1961E	0	0	2	4	0
L1970F	0	0	1	0	0
6519Δ11bp	0	0	0	1	0
D2177N	0	1	3	3	0
6568ΔC	0	0	0	0	1

Data are number of probands at each grade.

* The patient reported to have the R1129L *ABCR* variant in Table 1 of the original study[14] actually had Stargardt disease and should have been reported in the STGD column. Subsequent to the publication of the initial study, one of the original 167 patients was found to have a P940R *ABCR* variant that was not present in the STGD or general-population cohorts.

TABLE 3. Macular Characteristics of Initial Study Participants

Grade	*ABCR* Variant Present		*ABCR* Variant Absent		Total		Odds Ratio	95% Confidence Interval
	n	%	*n*	%	*n*	%		
2	1	3.8	5	3.5	6	3.6	8.6	0.5–159.8
3	11	42.3	45	31.9	56	33.5	10.5	1.3–84.9
4	13	50.0	48	34.0	61	36.5	11.6	1.5–92.8
5	1	3.8	43	30.5	44	26.3	1.0	—
Total	26	100	141	100	167	100		

to the probands, are of the same ethnicity, and have usually shared the same environment for decades, siblings are a reasonable population in which to study genotype–phenotype correlations of *ABCR* variants with AMD.[26] We attempted to enroll and to examine all available siblings, whether or not they had a known history of AMD. Self-reporting of a positive or negative history of AMD is notoriously inaccurate, especially because significant maculopathy (i.e., extensive drusen) can be compatible with normal visual acuity.[10,27] Among the fourteen Utah probands with *ABCR* variants, four had no living siblings and one declined to participate. The other nine Utah probands had a total of 27 living siblings, two of whom refused to participate. One other sibling was excluded due to the presence of confounding macular disease (bilateral parafoveal telangiectasis severe enough to have had laser treatment). Among the 12 Boston probands with *ABCR* variants, 2 had no siblings, 3 had no living siblings, and 1 declined to participate. The other six Boston probands had a total of nine living siblings, all of whom were willing to participate. Thus, a total of 33 living siblings from 15 families were available for examination.

Nineteen of these 33 siblings carried the family proband's variant *ABCR* allele. Fundus photograph grading of all available siblings revealed that exudative AMD (grade 5) was found in only two siblings, one with an *ABCR* variant, and one without an *ABCR* variant (Fig. 1). Thirteen of 19 siblings with the proband's variant had at least some signs of AMD (grades 2–5), whereas 6 had normal maculae. Eight of 14 siblings without the proband's variant had grades 2 to 5, presumably through a pathway not mediated by *ABCR*. Thus, siblings with the *ABCR* variant identical with the family proband were somewhat more likely to show signs of AMD when compared with the siblings who did not carry the variant (odds ratio 1.63; 95% confidence interval, 0.39–6.82). As discussed in the following paragraph, a much larger number of subjects are needed for sufficient power to confirm this trend in a statistically significant manner.

Statistical analysis becomes even more challenging if individual *ABCR* variants are examined, because the number of subjects becomes quite small, but two variants, G1961E and D2177N, deserve special attention. Not only are they more common than other AMD-associated variants,[14] but their association with risk of AMD has been confirmed in a large consortium study,[19] and they alter *ABCR* adenosine triphosphatase (ATPase) activity in vitro in a manner similar to the majority of Stargardt- and AMD-associated *ABCR* variants analyzed so far.[28] Three of four siblings of G1961E probands who also carried the variant G1961E allele had grade 2 or greater maculopathy, whereas both siblings who did not carry the variant allele had grade 4 disease. Four of seven siblings of D2177N probands who also carried the variant allele had grade 2 or greater maculopathy, whereas four of five siblings who did not carry the variant D2177N allele had grade 2 or greater maculopathy. Similar difficulty in demonstrating genotype–phenotype concordance in AMD families with these two *ABCR* variants has been reported recently.[29]

Pedigree analysis of the kindreds showed various patterns of association of the family proband's *ABCR* variant with AMD phenotype of the siblings. For example, all three living siblings in kindred K4495 share the proband's I1562T *ABCR* allele (Fig. 2), and all four had virtually identical severe bilateral geographic atrophy of the macula (grade 4 AMD) with onset late in the seventh or early in the eighth decade of life in each individual (Fig. 3). Kindred K4985, in contrast, shows no clear

TABLE 4. Characteristics of Initial Study Participants According to Presence or Absence of an *ABCR* Variant

	ABCR Variant Present		*ABCR* Variant Absent		Total		Odds Ratio	95% Confidence Interval
	n	%	*n*	%	*n*	%		
Age at diagnosis (y)								
≤65	9	35	47	34	56	34	1.6	0.5–5.7
66–75	13	50	60	43	73	44	1.8	0.6–6.1
≥76	4	15	34	23	38	22	1.0	—
Gender								
Female	17	65	98	70	115	69	0.8	0.3–2.0
Male	9	35	43	30	52	31	1.0	—
Family history of AMD								
Yes	16	62	66	47	82	49	1.8	0.8–4.3
No	10	38	75	53	85	51	1.0	—
Grade of AMD in each eye*								
Same	21	84	90	64	111	67	2.9	1.0–9.0
Different	4	16	50	36	54	33	1.0	—

* Two subjects had dense media opacities in the fellow eye.

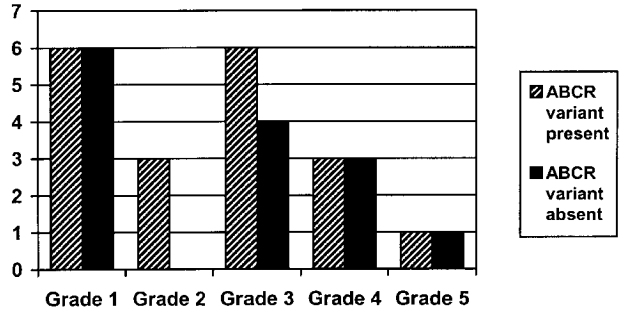

FIGURE 1. Distribution of AMD grade of siblings according to presence or absence of the proband's *ABCR* variant.

segregation of the family's D2177N *ABCR* allele with AMD phenotype (Fig. 4). The eldest sibling had moderate (grade 3) AMD, but did not carry the family proband's D2177N *ABCR* variant. Two siblings (one older and one younger than the proband) carried the D2177N variant and exhibited mild to moderate AMD, whereas two others (one older and one younger than the proband) carried the variant but exhibited no signs of AMD. It should be noted, however, that all members of this kindred were between 55 and 69 years old, and it is possible that AMD will develop in some of the unaffected siblings in future years, because the prevalence of AMD increases substantially after age 70.[31,32] The other 13 Utah and Boston pedigrees are displayed in Figure 5.

DISCUSSION

Age-related maculopathy and age-related macular degeneration are common traits that result from the complex interaction of environmental and hereditary risk factors. Some risk factors, such as cigarette smoking and increasing age, are well established by multiple epidemiologic studies.[30–32] Other risk factors such as nutrition and race are strongly suspected to play a role, along with gender, light exposure, and cardiovascular disease, for which the supporting data are more equivocal.[31–33] Genetic susceptibility to visual loss from AMD has long been suspected, especially because many patients note that AMD seems to "run in the family." This notion is supported by an epidemiologic study that demonstrated an age-adjusted 2.4-fold elevated relative risk of AMD in first-degree relatives of patients with AMD.[27] Definitive demonstration of an inherited risk factor for AMD has been elusive, however. Human molecular genetics has had by far the most success in defining the genetic etiology of ophthalmic diseases with a clear monogenic hereditary basis and well-defined diagnostic criteria. Genetic studies on retinitis pigmentosa,[34] retinoblastoma,[35] and color blindness[36] are excellent examples of such successes. Determining genetic factors involved in late-onset common ophthalmic disorders with variable clinical presentations such as glaucoma and AMD has been a far more evanescent goal.

A cornerstone methodology for studying the genetics of human diseases is the collection and characterization of multigenerational kindreds with the disorder. This approach is quite problematic for AMD, however. As a disease of the elderly, the affected proband's parents are usually dead, siblings are often dead or in widely scattered locations, and offspring are typically too young to manifest symptoms. Even with the ascertainment of a large kindred with AMD, the researcher is faced with substantial challenges, due to the high prevalence of the disease, its variable expressivity, and its apparent multifactorial etiology. Unlike a relatively uncommon disease such as retinitis pigmentosa, in which affected family members almost certainly have identical genetic defects, the situation is not as simple for AMD. Interaction of multiple AMD-associated alleles in many genes may be needed for increased susceptibility within a particular family. Thus, it cannot be assumed that affected siblings must have the same AMD-associated allele. The broad spectrum of clinical presentation of AMD ranging from exudative changes to geographic atrophy raises the question of whether AMD is truly one disorder or actually represents a multitude of diseases with different genetic etiologies, and the variable presentation and progression of AMD often requires an arbitrary delineation of which individuals are or are not affected. The interplay of nongenetic risk factors for AMD, such as smoking history, nutritional status, and light exposure complicates genetic studies because even if an individual has inherited a putative AMD susceptibility allele, the disease may not manifest if a protective lifestyle has been practiced. Also, age must be considered a factor, because a few soft and hard drusen in the macula of a 95-year-old patient may be normal, whereas the same findings in a 45-year-old patient may be considered the first signs of AMD.

Even if linkage is established to a chromosomal locus, it is often a long and arduous task to determine the actual genetic defect, because the chromosomal locus may encompass doz-

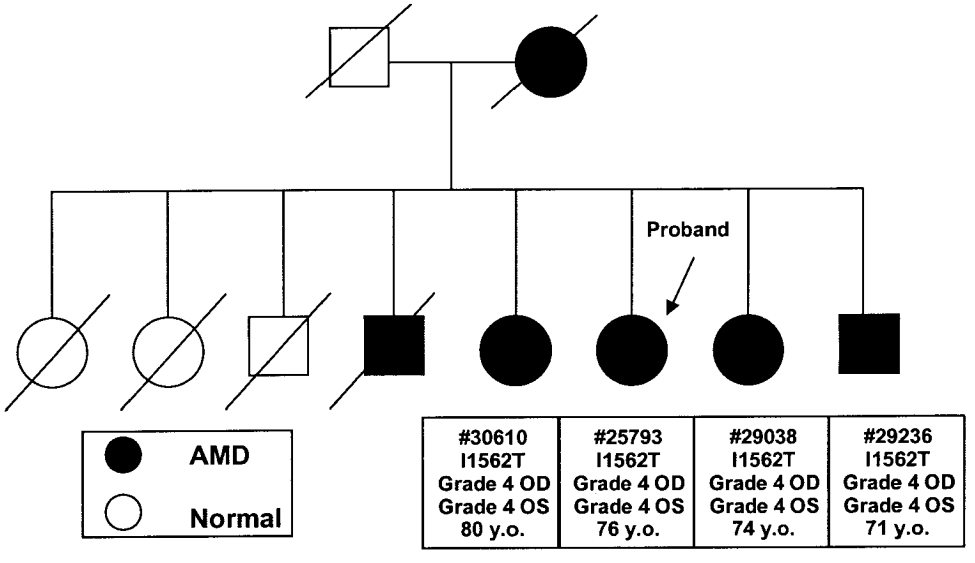

FIGURE 2. Pedigree of kindred K4495.

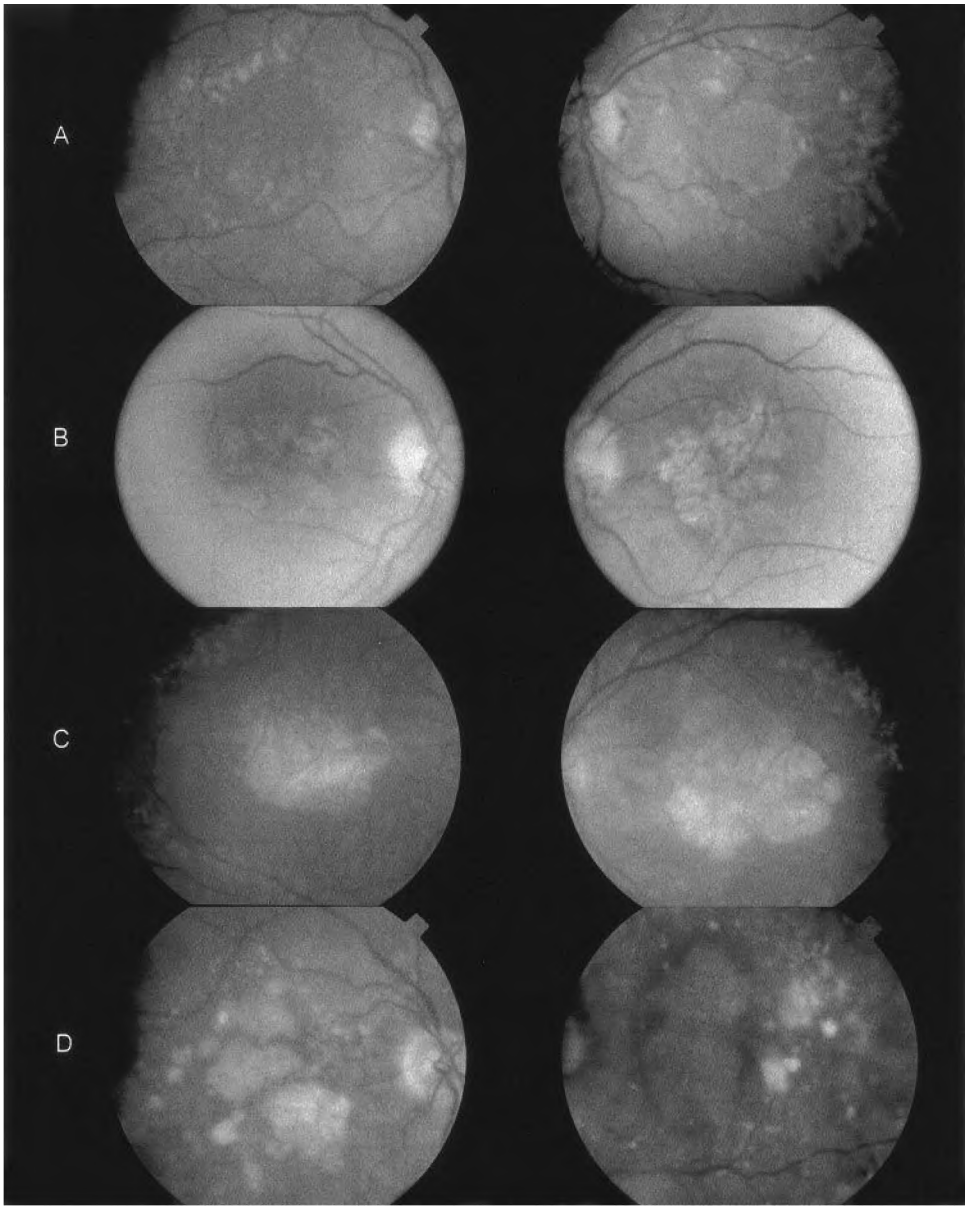

FIGURE 3. Fundus photographs from the family in Figure 2: (**A**) subject 30610: (**B**) subject 25793 (proband): (**C**) subject 29038: (**D**) subject 29236.

ens of genes. Significant linkage has been reported only recently in one AMD family at locus 1q25-31,[37] and it is likely that further progress with this approach will continue to be slow.[38]

The genetic investigation of AMD is amenable to the "candidate disease" approach. AMD shares phenotypic similarities to a number of hereditary diseases of the macula, and as the genetic bases for these diseases are ascertained, cohorts of patients with AMD can then be screened to determine whether comparable mutations are involved in the pathogenesis of AMD.[10] Stargardt disease (STGD1) is the most promising candidate disease for AMD. This autosomal recessive disorder is the most common early-onset macular dystrophy encountered in clinical practice (estimated frequency, 1 in 10,000).[4] It is characterized by macular atrophy and drusen-like flecks with associated central visual loss that typically occurs in the second or third decade of life but with earlier and later onsets well documented. The retinal pigment epithelium (RPE) accumulates large enough amounts of lipofuscin to exhibit a dark choroid on fluorescein angiography. Exudative complications are rare.[10]

If mutations in both alleles of *ABCR* can lead to protein dysfunction severe enough to manifest as an early-onset macular dystrophy such as STGD1, is it possible that a mutation in one *ABCR* allele could lead to moderate dysfunction sufficient to cause late-onset macular degeneration such as AMD? Our findings of an elevated frequency of amino acid–changing *ABCR* variants in patients with AMD relative to age-matched control subjects supports this hypothesis.[14,19] Physiologically, this hypothesis is tenable. In patients with STGD1, severe *ABCR* dysfunction disrupts vitamin A transport pathways from the outer segment disks leading to formation of massive amounts of lipofuscin, which accumulates in the RPE.[2] Less profound disruption of *ABCR* function in the heterozygous state acting over a prolonged period could lead to a similar accumulation of lipofuscin, albeit at a much slower rate. Indeed, lipofuscin formation is strongly associated with the progression of AMD,[39,40] and knockout mouse studies have confirmed that both homozygous and heterozygous mutations in *ABCR* are associated with increasing lipofuscin accumulation over time, especially when these animals are exposed to light.[41–43]

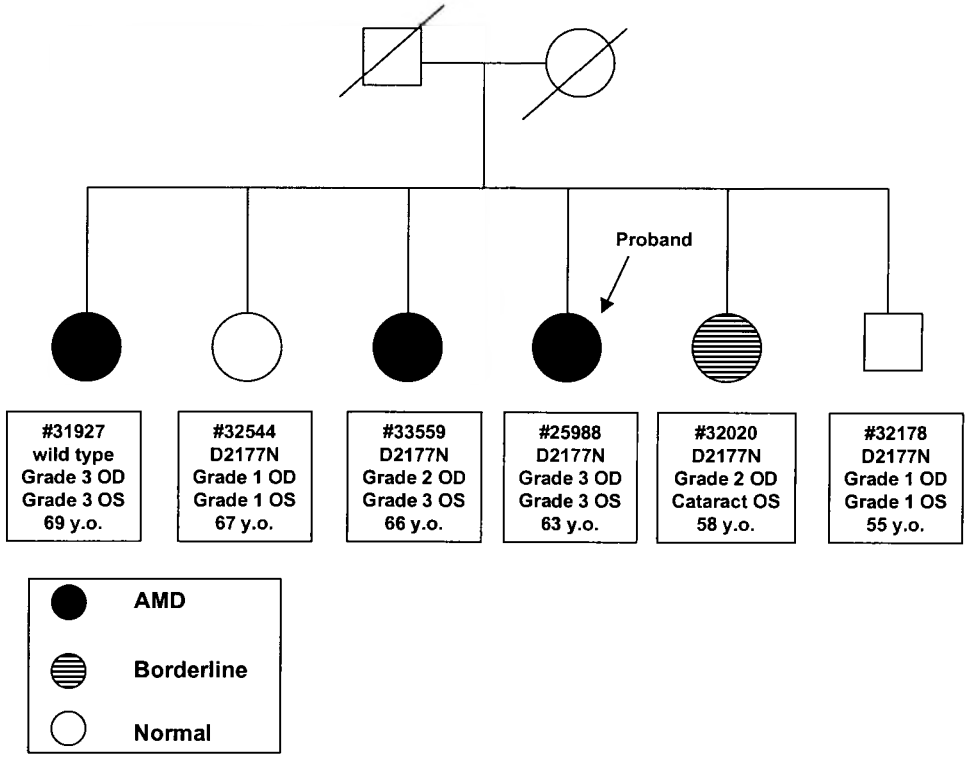

FIGURE 4. Pedigree of kindred K4985.

Similarly, as has been demonstrated in other clinical disorders associated with mutations in *ABCR*, this study was not and could not be designed to detect complex alleles that comprise a substantive fraction of all *ABCR* mutations, especially because complete sequencing and segregation through at least two generations could not be performed. However, complex alleles probably play a greater role in both the structural and physiologic functions of *ABCR* than have been appreciated to date, and the consequences of a single complex mutation in the heterozygote over many decades are only now being investigated in detail.[4,44,45]

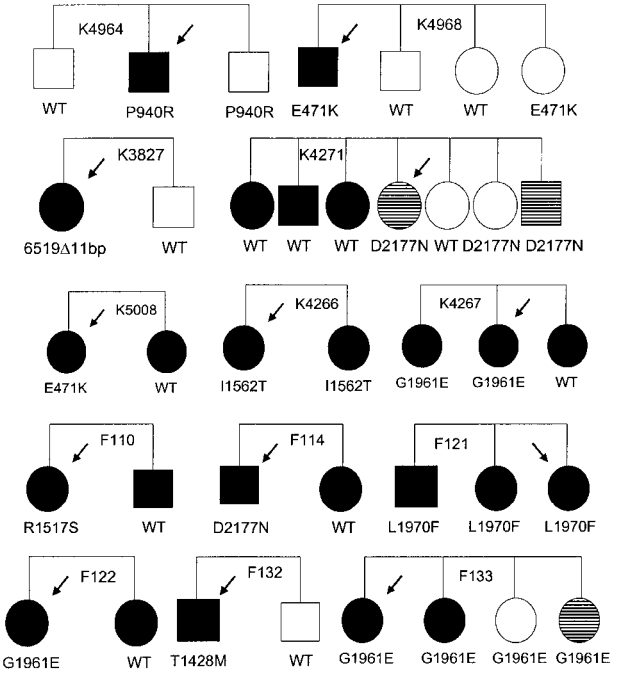

FIGURE 5. Pedigrees of 13 additional Utah (K) and Boston (F) *ABCR* AMD kindreds. *Arrows*: family proband for each kindred. The proband's *ABCR* variant is listed *below* each individual if it is present. WT, the sibling possesses the wild-type *ABCR* alleles, rather than the proband's variant. *Filled symbols*: grade 3 to 5 maculopathy present in at least one eye; *striped symbols*: borderline (grade 2) maculopathy; *open symbols*: no maculopathy in either eye (grade 1).

When we examined siblings of patients with *ABCR* variants, we demonstrated concordance of *ABCR* genotype with AMD phenotype in some families, but not in others. Also, when the data from all the families were pooled, they did not show a statistically significant correlation between *ABCR* variants and risk of AMD, possibly in part because of the relatively small numbers of study participants. Some elderly siblings had an *ABCR* variant, but no evidence of AMD. Other elderly siblings had AMD without having the same *ABCR* variant as the affected proband.

There are several possible explanations for the variable expressivity of AMD among those with *ABCR* variants. AMD progression in individuals with *ABCR* variants may be strongly influenced by concomitant environmental risk factors, such as smoking, light exposure, and diet, that were not examined or controlled in this study. Our study of the D2177N and G1961E mutations in age-matched ophthalmoscopically examined control subjects confirms that an *ABCR* variant does not by itself confer an AMD phenotype in all cases, but may increase susceptibility to the complex trait when large populations are examined.[19]

The fact that many siblings have AMD without the same *ABCR* variant as the family proband is not unexpected, especially because there are likely to be other inherited and environmental risk factors that have not yet been identified that may act alone or in concert with *ABCR* alleles to enhance susceptibility to AMD. Depending on the age of the individual, the risk of having AMD can be quite substantial. If an individual is over age 70, he or she has a 30% risk of having AMD or ARM.[31,32] Also, we did not screen for other *ABCR* mutations in

the siblings beyond the known variant of the proband. Thus, the contribution of other possible *ABCR* variants in these families is unknown. Because at least 4% of the general population is thought to carry a mutant *ABCR* allele,[5,18] this effect may be important.

The combined effects of variable expressivity at the age of surveillance and high disease prevalence made it unlikely that statistical significance could be achieved in a study of this size. This is a recurring problem facing investigators studying other complex adult-onset multifactorial diseases, such as breast cancer and prostate cancer.[46,47] Statistical power analysis indicates that we would need 144 siblings to achieve an 80% power of detecting a statistically significant elevated risk at $P = 0.05$ if the study population prevalence of AMD is assumed to be 10% and the elevated risk of AMD conferred by any AMD-associated *ABCR* variant is comparable to the approximately threefold elevation in AMD risk found for the G1961E and D2177N *ABCR* variants in the International *ABCR* Consortium Study.[19]

Although there is mounting evidence that heterozygous variants in *ABCR* contribute to AMD susceptibility, we should not expect consistent concordance of variant alleles with AMD phenotype, because it is a complex trait influenced by a multitude of other hereditary and environmental risk factors. Nevertheless, study of the families reported here has yielded important conclusions: (1) Affected siblings with the same *ABCR* variant may have highly concordant disease phenotypes. For example, all four siblings in kindred K4495 carried the same I1562T mutation, and all four had severe geographic atrophy in their eighth decade. (2) Exudative AMD, although present in more than 25% of the original study participants, was rare (<4%) among family probands found to have *ABCR* variants. Similarly, exudative AMD was also found to be uncommon (1/19) in probands' siblings possessing the identical *ABCR* variant. This correlates well with fact that Stargardt disease is almost exclusively a nonexudative macular dystrophy.

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