

# Cosegregation and functional analysis of mutant *ABCR* (*ABCA4*) alleles in families that manifest both Stargardt disease and age-related macular degeneration

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**Mutations in *ABCR* (*ABCA4*) have been reported to cause a spectrum of autosomal recessively inherited retinopathies, including Stargardt disease (STGD), cone-rod dystrophy and retinitis pigmentosa. Individuals heterozygous for *ABCR* mutations may be predisposed to develop the multifactorial disorder age-related macular degeneration (AMD). We hypothesized that some carriers of STGD alleles have an increased risk to develop AMD. We tested this hypothesis in a cohort of families that manifest both STGD and AMD. With a direct-sequencing mutation detection strategy, we found that AMD-affected relatives of STGD patients are more likely to be carriers of pathogenic STGD alleles than predicted based on chance alone. We further investigated the role of AMD-associated *ABCR* mutations by testing for expression and ATP-binding defects in an *in vitro* biochemical assay. We found that mutations associated with AMD have a range of assayable defects ranging from no detectable defect to apparent null alleles. Of the 21 missense *ABCR* mutations reported in patients with AMD, 16 (76%) show abnormalities in protein expression, ATP-binding or ATPase activity. We infer that carrier relatives of STGD patients are predisposed to develop AMD.**

## INTRODUCTION

Age-related macular degeneration (AMD) accounts for more than half of severe visual impairment in studied populations of European descent, with at least 1.7 million individuals affected in the USA. AMD is historically divided into two clinical subtypes: (i) atrophic or 'dry' AMD, characterized by accumulation of drusen in and under the retinal pigment epithelium (RPE) and atrophy of the macular retina and RPE; and (ii) exudative or 'wet' AMD, typified by invasion of

abnormal blood vessels into the subretinal space from the choroid and subsequent disciform degeneration.

This common disorder has been associated with both environmental factors (e.g. smoking) and genetic factors. Twin studies have shown a higher concordance of AMD phenotypes among monozygotic (~100%) than dizygotic (~40%) twins (1). Both clinic-based and population-based family studies have reported an increased risk of AMD among first-degree relatives of affected individuals (2,3). Family linkage studies have identified one possible locus for AMD on chromosome 1q (4).

As one genetic approach to macular degeneration, we isolated the gene for autosomal recessive Stargardt macular dystrophy (5). This gene, *ABCR* (also called *ABCA4*) encodes a photoreceptor-specific ATP-binding cassette transporter of retinaldehyde or a related derivative essential to the visual cycle. We and others have reported a spectrum of autosomal recessive retinal dystrophies associated with mutations in *ABCR*, including retinitis pigmentosa (RP19; 6–9), combined cone-rod dystrophy (7,10) and Stargardt disease/Fundus Flavimaculatus (STGD/FFM; 11–15). Viewed together, we and others have proposed a model in which retinal disease severity is correlated inversely with residual *ABCR* activity (16–18).

We reported previously an association between clinical AMD and heterozygous mutations in the STGD gene, *ABCR* (19). A subsequent multicenter international study confirmed this initial association for two specific disease-associated variants (G1961E and D2177N) and estimated a 3–5-fold increased risk for development of AMD among carriers of these two *ABCR* mutations (20). However, controversy regarding our initial findings (21) and the failure of other studies to replicate that observation (22–24) necessitated a test for the association of *ABCR* mutations with AMD by an independent approach.

We suggested previously a study of families that manifest both STGD and AMD (17). In this approach, a variation of the index-test method reported by Swift *et al.* (25,26), STGD-affected individuals are screened for mutations in *ABCR*, and

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subsequently these mutations are tested for cosegregation with both STGD and AMD in each family (17). Importantly, only one AMD-affected from each side of the family is tested to minimize potential false positives due to either linked AMD-associated loci or mutations at other AMD-associated loci manifesting disease within a family. Other investigators tested the association of STGD alleles with AMD in one North American and three French families, but this small cadre of families was insufficient to estimate the potential risk of AMD in STGD carriers for these few mutations (17,27).

Here, we present complete sequence analysis of *ABCR* and tests of cosegregation in 22 families that manifest both STGD and AMD, selected from our cohort of over 300 STGD families (13). We also analyzed functional properties of AMD-associated *ABCR* mutations by testing protein expression and ATP binding in an *in vitro* assay. Taken together, these data show that STGD-causing mutations in *ABCR* are associated with AMD and that many AMD-associated mutant *ABCR* proteins have substantive defects in both expression and ATP binding.

## RESULTS

### *ABCR* mutation analysis

Twenty-two families affected with recessive Stargardt macular dystrophy reported a history of AMD in at least one relative who was not an obligate carrier (e.g. grandparent; Fig. 1). STGD was diagnosed in the proband by previously published criteria that included a dark choroid on fluorescein angiography (13). Ophthalmic records including fundus photographs and fluorescein angiograms were reviewed for each reported AMD-affected individual to confirm the diagnosis. Individuals with other age-dependent causes of visual loss were excluded from analysis, including diagnoses of ischemic optic neuropathy, diabetic retinopathy, retinal vascular occlusion, foveal hole formation and epiretinal membranes. We sequenced all exons and flanking intronic regions of *ABCR* for all STGD probands, independent of any mutation analysis reported previously in these families. Complete *ABCR* sequencing was also performed for individuals AR33-8 and AR468-8 to characterize all STGD alleles in these two multi-generation affected families (Fig. 1; Table 1). We established the segregation of each identified *ABCR* mutation by sequencing the corresponding exon in all available family members. This segregation analysis assigned the carrier status of each AMD-affected relative. One hundred control chromosomes were screened for all novel nucleotide alterations identified by our sequence analysis of STGD probands.

*ABCR* mutations were identified by this direct sequencing method in 37/46 (80%) STGD disease chromosomes (Table 1; Fig. 1). In 15/22 (68%) STGD families, probands were compound heterozygous or homozygous for *ABCR* mutations, 5/22 (23%) were heterozygous and 2/22 (9.1%) had no identified *ABCR* mutations. Of note, AR468-8 was heterozygous for three mutations: the transitions 3758C→T (encoding the missense mutation T1253M), 4139C→T (encoding the missense mutation P1380L) and 5882G→A (encoding the missense mutation G1961E). Segregation analysis in this family revealed that two alterations (T1253M and G1961E) were on the same chromosome; thus AR468-8 is compound heterozygous for a novel complex allele [T1253M; G1961E]

and the missense mutation P1380L (Fig. 1). AR33 and AR215 segregate complex alleles as well: the mutant allele [W1408R; R1640W] was observed in all affected siblings of the proband AR33-1 and also both the AMD-affected mother and AMD-affected maternal aunt (Fig. 1). The complex allele [H1406Y; V2050L] was identified in STGD-affected AR215-4 (Fig. 1).

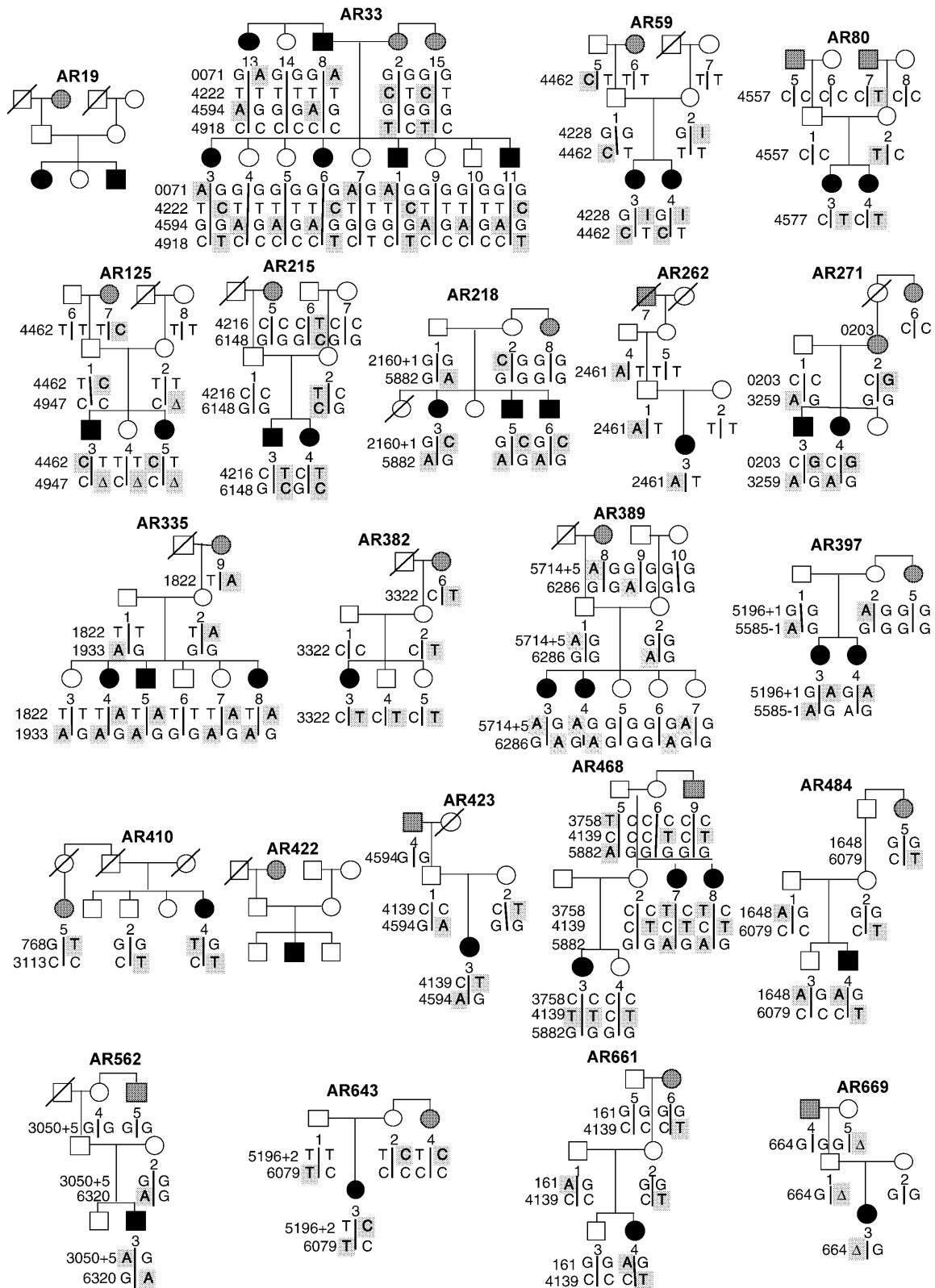
Two novel mutations were identified in this cohort: the missense mutation T1253M was identified as part of the complex allele [T1253M; G1961E] and the transition 1648G→A (encoding the missense mutation G550R) was identified in STGD-affected AR484-4. Neither of these mutations was identified in 100 control chromosomes. All other mutations identified in these pedigrees have been reported previously. The complex allele [W1408R; R1640W] was reported recently in an unrelated family segregating both STGD and retinitis pigmentosa (9).

### Analysis of AMD relatives of STGD affecteds

To test the hypothesis that AMD-affected relatives are more frequent carriers of STGD alleles, we compared the proportion of AMD-affected carriers observed in our cohort to that predicted by the null hypothesis. In each of these 22 families, the AMD-affected has an a priori likelihood of being a carrier of a STGD allele based upon his or her position within the pedigree. (The exact probability of being a carrier is the sum of the a priori probability and the population carrier frequency of the mutation being tested; because these small population carrier frequencies are unknown, we used only the a priori values in this test.) We used the single proportion *Z* test to compare the proportion of carriers observed in our cohort to that predicted by the null hypothesis. We tested our hypothesis in 19 of the 22 families reported here (Fig. 1; Table 1). In three families (AR19, AR215, AR422) mutations were not identified which could be tested for segregation with AMD. Our analysis also included family AR534 (17) and families 1–3 from another source (27), each of which also show both STGD and AMD. For these 23 families,  $P_{\text{predicted}} = 10.25/23 = 0.446$  (Table 1). Cosegregation of the STGD-associated *ABCR* mutant allele was observed in 15/23 cases, therefore  $P_{\text{observed}} = 0.652$ ;  $Z = 2.07$ ,  $P = 0.038$ . Thus, AMD-affected relatives of STGD probands are significantly more likely to be carriers of *ABCR* mutations than expected by random assortment of these alleles within the families.

### Biochemical analysis of recombinant *ABCR*

To investigate further the role of *ABCR* mutations in AMD, we tested AMD-associated *ABCR* mutations that were not functionally analyzed previously for defects in protein expression and ATP binding (19,28). Plasmids that express the *ABCR* cDNA under control of the cytomegalovirus promoter were constructed and mutagenized to contain the point mutations identified in patients with AMD. Plasmids were transiently transfected into HEK 293T cells. Thirty six to 42 h after transfection, the cells were harvested and both RNA and protein were purified. To confirm expression of the mRNA, dot blots of total RNA were hybridized with both *ABCR* and  $\beta$ -actin probes. We measured transfection efficiency by the ratio of *ABCR* to  $\beta$ -actin signal intensity. This analysis showed that each mutant construct expressed abundant *ABCR* mRNA (data not shown).



**Figure 1.** Pedigrees cosegregate *ABCR* mutations with both STGD and AMD. Pedigrees are drawn with standard conventions; STGD-affected individuals are filled symbols; AMD-affected individuals have diagonal cross bars. Pedigree numbers are shown above each drawing; individual numbers are shown below each symbol. *ABCR* mutation genotypes are shown on each side of a vertical bar below the individual identifier number. Nucleotide positions are shown at left and sequences at these positions shown below each individual. Mutations are indicated in bold; background shading, I, insertion; Δ, deletion. See Table 1 and the text for corresponding amino acid changes.

**Table 1.** *ABCR* mutations in families with both STGD and AMD

Pedigree	Maternal allele	Paternal allele	AMD relative	A priori	Cosegregation
AR19			pGM, -6	0.5	-
AR33	<b>[W1408R; R1640W]</b>	R24H and D1532N	mA, -16	0.5	Yes
AR59	4232insTATG	<b>C1488R</b>	pGM, -6	0.5	No
AR80	T1526M		pGF, -5	0.5	-
AR80	<b>T1526M</b>		mGF, -7	0.5	Yes
AR125	4947delC	<b>C1488R</b>	pGM, -7	0.5	Yes
AR215	[H1406Y; V2050L]		pGM, -5	0.5	-
AR218	<b>2160+1G→C</b>	G1961E	mA, -8	0.5	No
AR262		<b>W821R</b>	pGGF, -7	0.25	No
AR271	<b>P68R</b>	E1087K	mGA, -6	0.25	No
AR335	<b>D645N</b>	F608I	mGM, -9	0.5	Yes
AR382	<b>R1108C</b>		mGM, -6	0.5	Yes
AR389	E2096K	<b>5714+5G→A</b>	pGM, -8	0.5	Yes
AR397	<b>5196+1G→A</b>	5585-1G→A	mA, -5	0.5	No
AR410	A1038V	<b>768G→T</b>	pC, -5	0.25	Yes
AR422			pGM, -6	0.5	-
AR423	P1380L	<b>D1532N</b>	pGF, -4	0.5	No
AR468	<b>P1380L</b>	P1380L	mU, -9	0.5	Yes
AR484	<b>L2027F</b>	G550R	mGU, -5	0.25	Yes
AR562	R2107H	<b>3050+5G→A</b>	pGU, -5	0.25	No
AR643	<b>5196+2T→C</b>	L2027F	mU, -4	0.5	Yes
AR661	<b>P1380L</b>	C54Y	mGF, -6	0.5	Yes
AR669		<b>664del13</b>	pGF, -4	0.5	No
AR534	W821R	<b>P1380L</b>	pGM, -7	0.5	Yes (17)
Family 1	<b>R212C</b>	I2113M	mGM, I-2	0.5	Yes (27)
Family 2	<b>R1108C</b>	R2107H	mGM, I-2	0.5	Yes (27)
Family 3	<b>R212C</b>	G1977S	mGF, I-1	0.5	Yes (27)
				10.25	15

Standard mutation nomenclature is used. The relationship of the AMD-affected to the STGD proband and individual number is indicated in the column 'AMD relative': p, paternal; m, maternal; U, uncle; A, aunt; C, cousin; GF, grandfather; GM, grandmother; GGF, great grandfather. (Fig. 1). A priori indicates the approximate chance that the AMD-affected relative would be a carrier of a STGD allele based on their position within the pedigree. The column titled 'Cosegregation' indicates whether the AMD relative was a carrier confirmed by sequence results. The four pedigrees listed at the bottom are from the previous studies by Shroyer *et al.* (17) and Souied *et al.* (27). Totals for the last two columns are given at the bottom. Alleles that were tested for cosegregation are in bold.

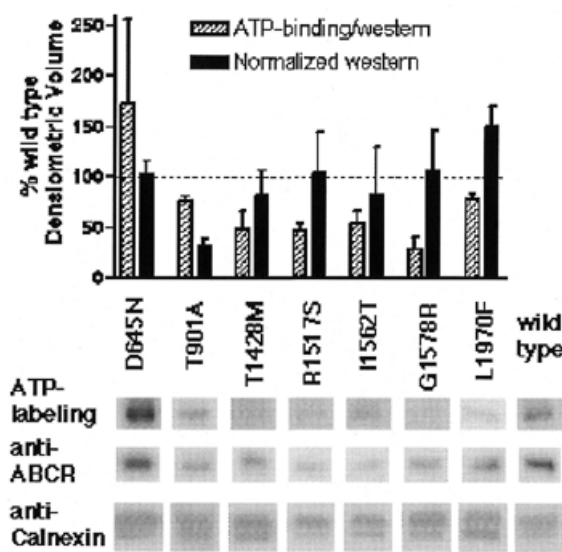
We performed western blots with both total protein and purified 293T membranes from cells transfected with mutant *ABCR* plasmids. Western blots were probed with both the anti-*ABCR* antibody Rim3F4 and the anti-calnexin antibody SPA-860 (as a loading control). *ABCR* immunoreactivity was normalized to that of calnexin and compared with the transfection efficiency from the northern blots (Fig. 2). This analysis showed that some AMD-associated mutations confer mild to moderate defects in expression or stability of the nascent *ABCR* protein.

Total membranes from transfected 293T cells were labeled with [ $\alpha$ - $^{32}$ P]-8-azido-ATP to assess the ATP-binding ability of selected AMD-associated *ABCR* mutations (Fig. 2). ATP-labeling was quantitated by densitometry and compared to western blots of the same membranes as one measure of normalized

ATP binding (Fig. 2). In this analysis, substantive defects exist for six of seven tested AMD-associated *ABCR* mutations. The seventh mutation, D645N, had an increased affinity for ATP determined by these ATP-labeling experiments.

## DISCUSSION

We screened for *ABCR* mutations by direct sequence analysis in 22 families that expressed both STGD and AMD. We identified mutations in 37/46 (80%) STGD chromosomes, a rate that is substantially higher than the best reported by mutation scanning methods (63%; 12). Whereas this mutation detection rate is an improvement over previous reports, a large fraction of alleles remain uncharacterized. Large deletions spanning more than one exon have been reported (8,12) but are



**Figure 2.** ATP-labeling and western blots of recombinant *ABCR*. The filled bars show *ABCR* immuno-reactivity normalized to RNA levels as determined by dot-blot hybridization. Bars with diagonal cross-hatches show ATP-labeling intensity normalized to *ABCR* immunoreactivity. Each bar is plotted as the fraction of wild-type and error bars represent the SE ( $n = 3$  for each mutant construct). Typical results of an *ABCR* ATP-labeling experiment are shown with the corresponding anti-*ABCR* and anti-calnexin western blots below the bar graph for each mutant construct (a wild-type control sample is shown at right).

unlikely to account for many of the remaining alleles (our unpublished observations). Thus, mutations that lie outside of the coding regions of the *ABCR* gene probably account for some fraction of disease alleles. Alternatively, presumed benign alterations may be pathogenic when combined with other alterations in a complex allele; evidence for a synergistic effect of two mutations in a complex allele has been reported recently for the [W1408R; R1640W] allele (9).

Two case-control studies have reported mutations in *ABCR* associated with AMD. One study that used a mutation screening method identified heterozygous *ABCR* mutations in 26/167 (16%) of unrelated subjects with AMD (19). A second multicenter international study assessed the frequency of two common *ABCR* mutations in 1218 AMD patients and 1258 control subjects, and reported a significantly higher prevalence of mutations in cases than controls (20).

Nonetheless, this reported association of *ABCR* mutations with AMD has been contested (21–24), despite differences in both testing algorithms and study procedures and populations. Therefore, we had proposed an alternate strategy to test for the association of *ABCR* mutations with AMD by assessing the segregation of mutant alleles among families that manifest both STGD and AMD (17).

Here we report the statistically significant association of *ABCR* mutations among individuals with AMD with this segregation method. Fifteen out of 23 (65.2%) families segregate STGD-associated *ABCR* mutations with AMD-affected relatives, whereas we expected 44.6% of relatives to be carriers by chance alone. Whereas this association is significant ( $P < 0.05$ ), analysis of additional families with both STGD and AMD is warranted to confirm and to extend these findings, and

to measure the relative risks to individual carriers of specific *ABCR* mutations to develop AMD. For example, the mutation P1380L was identified in 3/3 AMD-affected relatives of STGD patients (AR468-9, AR534-7, AR661-6). Although not significant ( $P = 0.083$ ) by itself, analysis of additional STGD families who share the P1380L allele may reveal a selective association with AMD and thus allow calculation of the risk for this allele. Similarly, this survey attended only the ophthalmic disorders and the molecular mechanisms, and was not intended nor designed to evaluate environmental or epidemiological cofactors in the evolution of AMD. Furthermore, analysis of pedigrees that manifest AMD and other *ABCR*-associated recessive retinopathies (e.g. retinitis pigmentosa or cone-rod dystrophy) may also be informative: do mutations that cause these severe retinopathies also predispose to AMD in the heterozygous state?

Together with these data, 27 different *ABCR* mutations have been associated with AMD (19,27,29). Some mutations identified in patients with AMD have been biochemically characterized previously. Sun *et al.* (28) reported substantial defects in protein expression or ATP binding of eight AMD-associated mutations (R212C, G863A, A1038V, R1108C, R1129L, P1380L, G1961E and L2027F) and an abnormal increase in the ATPase activity of the D2177N mutation, and they reported mild defects or wild-type activity within the sensitivity of the assay in four other AMD-associated variants (E471K, C1488R, T1526M and R1898H). Furthermore, the AMD-associated complex allele [W1408R; R1640W] has been reported to cause a severe defect in protein expression and ATP binding (9).

To analyze the function of AMD-associated *ABCR* mutations, we characterized the effects of seven different missense mutations (D645N, T901A, T1428M, R1517S, I1562T, G1578R and L1970F) on protein expression and ATP binding. We found that six of these mutations substantially reduced the ATP-binding ability of the mutant protein, whereas the mutation D645N showed no apparent defect compared to wild-type and actually had an increased affinity for ATP (Fig. 2). These results are intriguing because none of the tested mutations is within either the Walker A or B nucleotide-binding domains (the alteration L1970F is adjacent to the second Walker A motif). Thus, our results may suggest either that these mutations cause misfolding of this protein or that the transmembrane or intradiskal loop domains are somehow important in regulating the ATP-binding activity of *ABCR*.

Our data, taken with those of Sun *et al.* (28) and with the results on the [W1408R; R1640W] allele (9), demonstrate that 16/21 AMD-associated missense *ABCR* mutations manifest an abnormal effect on either protein expression, ATP-binding or ATPase activity. The finding of minimal or no defects for some mutations with this *in vitro* functional assay does not necessarily reflect normal activity for these mutant proteins; no measurements of transport of a putative substrate (e.g. retinaldehyde) were made, nor were these mutant proteins assessed in a normal physiological environment (e.g. a photoreceptor). Nonetheless, these results strengthen the association of *ABCR* mutations with AMD by demonstrating functionally evident defects in most disease-associated variants, even with this limited assay system. The hypothesis that heterozygous *ABCR* mutant alleles may predispose to AMD is further supported by animal studies. Recent work by Mata *et al.* (30) document a time- and light-dependent accumulation of A2E (a conjugate of

retinaldehyde and phosphatidylethanolamine that is the major constituent of lipofuscin seen in the drusen of AMD patients) in heterozygous *Abcr* +/- mice.

Further analysis of *ABCR* in an independent cadre of AMD-affected individuals will confirm and extend these findings. Hundreds of pedigrees with recessive *ABCR*-associated retinopathies have been reported and many have yet to be evaluated. Ophthalmic counseling and monitoring of older carrier relatives and at-risk individuals in these families may be justified because of increased risk for AMD.

## MATERIALS AND METHODS

### Patients

Families affected with classical recessive STGD were previously polled for a family history of AMD (13). Of 145 families polled, 21% (30/145) reported AMD in a relative of the STGD proband. Additional families with both STGD and AMD were enrolled by referral for evaluation of STGD, and subsequent report of a family history of AMD. Clinical records on all reported AMD-affected subjects were reviewed to confirm the diagnosis and stage of disease. For individuals with confirmed diagnoses of AMD, blood was obtained for molecular analyses.

AMD was defined as ophthalmoscopically identifiable macular pathology, including macular drusen, RPE detachment, geographic atrophy, exudative AMD including RPE detachment, choroidal neovascularization or disciform scar (31). Individuals were excluded if they had other causes of disciform or exudative maculopathy similar to AMD, such as ocular histoplasmosis syndrome, myopic chorioretinal degeneration without or with lacquer cracks, pseudoxanthoma elasticum and other associations of angioid streaks, basal laminar drusen or post-traumatic choroidal ruptures. Also excluded were individuals with any other retinal and retinal vascular pathology (retinitis pigmentosa, vascular occlusive disease or diabetic retinopathy). STGD was diagnosed according to previously published criteria (32) which included central visual impairment and a dark choroid on fluorescein angiography.

Controls were unrelated individuals over the age 65 years, unrelated to any known STGD family and who had normal binocular indirect and biomicroscopic examinations of the retina by a single observer (R.A.Lewis). These studies were approved by The Institutional Review Board for Human Subject Research at Baylor College of Medicine.

### *ABCR* genetic analysis

DNA was extracted from peripheral leukocytes by standard methods (33). We sequenced directly all 50 exons of *ABCR* (15). Briefly, tailed PCR amplification products of the exons and flanking intronic regions of *ABCR* were sequenced with BigDye M13-21 and reverse ready reaction kits (Applied Biosystems). Sequencing reaction products were analyzed on an ABI 377 automated sequencer (Applied Biosystems). For each exon in which a novel nucleotide alteration was identified, at least 100 control chromosomes were also sequenced. We analyzed all nucleotide alterations that were not identified in controls for segregation within each respective family by

direct sequencing of the corresponding exons in all available family members.

### Statistical analysis

We used the program WebStat (<http://www.stat.sc.edu/webstat/>) (34) for statistical calculations. The exact binomial test was used to compare the proportion of AMD-affected STGD allele carriers observed in our cohort to that predicted by the null hypothesis: the proportion of carriers predicted by random assortment of the STGD alleles within the pedigree (e.g. the null hypothesis predicts 50% of grandparents to be carriers of STGD alleles). There is no selection bias for this study since a priori one could not know the *ABCR* mutation status of the AMD-affected relative.

### *ABCR* mutant constructs

Plasmid pRK5-*ABCR* was provided generously by Dr Jeremy Nathans (28). Plasmid mutagenesis was performed with the QuickChange XL mutagenesis kit (Stratagene). Overlapping oligos (Table 2) were designed to incorporate the desired mutation into the mutagenized plasmid. Multiple mutant clones were sequenced to confirm the mutations and ensure no random alteration of the plasmid.

### Transfections and membrane protein purification

Mutant and wild-type pRK5-*ABCR* plasmids were transiently transfected into HEK 293T human embryonic kidney cells for expression of recombinant *ABCR* protein. In a typical experiment, four 10 cm dishes of cells were transfected each with 30 µg of plasmid DNA and 1.5 µl of Lipofectamine 2000 (Life Technologies) in 3 ml of serum-free media as described in the manufacturer's instructions. Cells were harvested and membranes isolated (28). Protein concentration of the 293T membranes was determined with the BCA kit (Pierce). For RNA analysis, 2 ml of Trizol (Life Technologies) was added directly to the cells in the culture dish. RNA and protein was isolated from Trizol homogenates according to the manufacturer's instructions.

### Western blotting and azido-ATP labeling

Total membrane proteins were separated by SDS-PAGE on 4–15% gradient gels (Bio-Rad). Proteins were transferred to PVDF membranes and blotted with anti-*ABCR* monoclonal antibody Rim3F4 (a gift from Dr Robert S.Molday) and anti-calnexin rabbit polyclonal antibody SPA-860 (StressGen).

[ $\alpha$ -<sup>32</sup>P]-8-azido-ATP was used to label ATP-binding membrane proteins (9,28,35,36). Under dim red light, [ $\alpha$ -<sup>32</sup>P]-8-azido-ATP was dried under an air stream and resuspended to 4 µM in Buffer I (25 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>). Membrane proteins of 2, 4 or 8 µg were diluted to 8 µl in Buffer I and an equal volume of [ $\alpha$ -<sup>32</sup>P]-8-azido-ATP was added. The reactions were incubated at room temperature for 5 min then irradiated with a handheld 302 nm UV light for 5 min at 10 cm. [ $\alpha$ -<sup>32</sup>P]-8-azido-ATP labeled membranes were separated by SDS-PAGE on 4–15% gradient gels, transferred to PVDF membranes, autoradiographed and blotted with Rim3F4 and SPA-860 antibodies as described above.

**Table 2.** Oligonucleotide sequences for *ABCR* mutagenesis

Nucleotide alteration	Amino acid change	Mutagenic oligonucleotide 5'→3'
1933G→A	D645N	CTGCTTCGTGGAC <u>A</u> ATTCTTTCATGATC
2701A→G	T901A	AGACCGAGCCCTA <u>G</u> CAGAGGAAACGG
4283C→T	T1428M	GGCAGTGAGCAGTTCA <u>T</u> GGTACTTGCAGACGTCCTC
4549C→A	R1517S	CCCAGAGAACACAG <u>A</u> GCAGCACGGAAATTCTACAAG
4685T→C	I1562T	GGAGGAATTTCCA <u>C</u> TGGAGGAAAGCTCC
4732G→A	G1578R	GGAAGCACTTGT <u>T</u> AGGTTTTTAAGCGAC
5908C→T	L1970F	GAGTGCTTTGGC <u>T</u> TCTCTGGGAGTGAATGG

Mutated bases are underlined and bold.

## RNA hybridization

For RNA blotting, 2 µg of total RNA from each transfection was treated with DNase I and spotted onto a nylon membrane (Oncor) with a Bio-Dot apparatus (Bio-Rad). The membranes were hybridized with probes to human β-actin (quantitation control) or a 1649 bp *Eco*RI restriction fragment from the *ABCR* cDNA corresponding to exons 7–16, washed and autoradiographed.

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