Null Missense ABCR (ABCA4) Mutations in a Family with Stargardt Disease and Retinitis Pigmentosa

Noah F. Shroyer,1,2,3 Richard Alan Lewis,2,4,5,6 Alexander N. Yatsenko,2 and James R. Lupski1,2,4

PURPOSE. To determine the type of ABCR mutations that segregate in a family that manifests both Stargardt disease (STGD) and retinitis pigmentosa (RP), and the functional consequences of the underlying mutations.

METHODS. Direct sequencing of all 50 exons and flanking intronic regions of ABCR was performed for the STGD- and RP-affected relatives. RNA hybridization, Western blot analysis, and azido-adenosine triphosphate (ATP) labeling was used to determine the effect of disease-associated ABCR mutations in an in vitro assay system.

RESULTS. Compound heterozygous missense mutations were identified in patients with STGD and RP. STGD-affected individual AR682-03 was compound heterozygous for the mutation 2588G→C and a complex allele, [W1408R; R1640W]. RP-affected individuals AR682-04 and 05 were compound heterozygous for the complex allele [W1408R; R1640W] and the missense mutation V767D. Functional analysis of the mutation V767D by Western blot and ATP binding revealed a severe reduction in protein expression. In vitro analysis of ABCR protein with the mutations W1408R and R1640W showed a moderate effect of these individual mutations on expression and ATP-binding; the complex allele [W1408R; R1640W] caused a severe reduction in protein expression.

CONCLUSIONS. These data reveal that missense ABCR mutations may be associated with RP. Functional analysis reveals that the RP-associated missense ABCR mutations are likely to be functionally null. These studies of the complex allele W1408R; R1640W suggest a synergistic effect of the individual mutations. These data are congruent with a model in which RP is associated with homozygous null mutations and with the notion that severity of retinal disease is inversely related to residual ABCR activity. (Invest Ophthalmol Vis Sci. 2001;42:2757–2761)

From the 1Program in Cell and Molecular Biology and the Departments of Molecular and Human Genetics, 2Pediatrics, 3Medicine, and 4Ophthalmology, Baylor College of Medicine, Houston, Texas.

Supported in part by Grant R01 EY11780 from the National Institutes of Health, the Milton and Ruth Steinbach Fund, the Foundation Fighting Blindness, and unrestricted funds from Research to Prevent Blindness to the Department of Ophthalmology, Baylor College of Medicine. NFS is supported by predoctoral training Grant T32 EY07102 from the National Eye Institute. RAL is a Senior Scientific Investigator of Research to Prevent Blindness.

Submitted for publication April 23, 2001; accepted July 10, 2001.

Commercial relationships policy: P.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1754 solely to indicate this fact.

Corresponding author: James R. Lupski, Baylor College of Medicine, 604B, One Baylor Plaza, Houston, TX 77030. jflupski@bcm.tmc.edu

Retinitis pigmentosa (RP) is a clinical diagnosis describing a group of genetically heterogeneous progressive retinal dystrophies caused by at least 29 genes (listed at http://www.sph.uth.tmc.edu/Retnet/, provided by the University of Texas Houston Health Science Center).1 Stargardt disease (STGD) is an autosomal recessive macular dystrophy, usually of juvenile onset, thought to be genetically homogeneous. ABCR (also known as ABCA4), the gene encoding a photoreceptor-specific adenosine triphosphate (ATP)-binding cassette transporter, is mutated in STGD1.2 Classically, STGD1 and RP have been viewed as clinically distinct disorders with no common genetic basis. However, after a locus for recessive RP (RP19) was mapped to the STGD1 interval on chromosome 1p, homozygous ABCR frameshift mutations were identified in a consanguineous Spanish isolate with RP19.3 An unrelated Dutch family that manifested both RP and cone–rod dystrophy was found to harbor splice-site mutations in ABCR.4 Recently, in a French family manifesting both STGD1 and RP19, a splice mutation was found in the STGD1-affected individual, and the RP-affected cousin was apparently hemizygous for this mutation.5 To date, all ABCR mutations associated with RP19 have been putative truncating alleles; no RP-associated ABCR missense mutations have been reported. Thus, some forms of recessive RP are caused by presumed null mutations of ABCR, and a spectrum of dystrophic retinal phenotypes is associated with various ABCR mutations.6–8

We present the clinical analyses of a family that manifests both STGD1 and RP, identify STGD- and RP-associated missense ABCR mutations, and describe functional biochemical studies of the disease associated alleles. These studies support the model relating retinal disease severity to the reduction in ABCR activity.

METHODS

Patients

This study was approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine. The study protocol adhered to the tenets of the Declaration of Helsinki. A North American family of European descent manifesting both STGD1 and RP was culled from a large cohort who volunteered for participation in the locus identification, gene cloning, and genotype-phenotype correlations of paired mutant alleles of STGD1. Enrollment criteria for the study of STGD have been published previously.2,9,10 RP was defined by criteria consonant with prior international standards1 and confirmed by review of all available ophthalmic records and retinal photographs. Incisive distinction between the two clinical diagnoses can be made by these differing criteria. DNA used as a control was obtained from unrelated North American individuals who had no history of retinal disease.

Genetic Analysis of ABCR

DNA was extracted from peripheral leukocytes by standard methods.11 Primers to the intronic regions flanking each exon of ABCR were tailored at their 5’ ends with M13 –21 or M13 reverse sequences.6 These tailed primers were used to amplify by PCR the exons and flanking intronic regions of ABCR in the STGD1 proband and an RP-affected individual.

Copyright © Association for Research in Vision and Ophthalmology

2757
We performed bidirectional dye primer sequencing (BigDye M13 -21 and reverse sequencing kits; PE-Applied Biosystems, Foster City, CA). Sequencing products were analyzed on an automated sequencer (ABI 377; PE-Applied Biosystems).

Nucleotide alterations were verified, and segregation analysis was performed by directly sequencing variant exons in every available family member. Control DNA was amplified and sequenced in the same manner.

In Vitro Analysis of ABCR

Plasmid pRK5-ABCR was provided by Jeremy Nathans (Johns Hopkins Medical School, Baltimore, MD).12 Plasmid mutagenesis was performed with a mutagenesis kit (QuickChange XL; Stratagene, La Jolla, CA). Overlapping oligos were designed to incorporate the desired mutation into the mutagenized plasmid. Oligos corresponding to each mutation were: V767D, GTC TGG CAG CAG CCT GTA GTG ACA TCT ATT TCA C; W1408R, GAC CCT TCA CCC CGG GAT ATA TGG GCA G; R1640W, CAA CGC CAT CTT A; CAA CGC CAT CTT A; and CAA CGC CAT CTT A.

In a typical experiment, four 10-cm dishes of cells were transfected each with 30 µg plasmid DNA and 1.5 µl lipofection reagent (Lipofectamine 2000; Life Technologies, Rockville, MD) in 3 ml serum-free medium, as described in the manufacturer’s instructions. Cells were harvested and membranes isolated as described.12 Protein concentration of the 293T membranes was determined with a BCA kit (Pierce, Rockford, IL). For RNA analysis, 2 ml RNA extraction reagent (TRIzol; Life Technologies) was added directly to the cells in the culture dish. RNA and protein were isolated from the reagent homogenates according to the manufacturer’s instructions.

For Western blot analysis, total membrane proteins were separated by SDS-PAGE on 4% to 15% gradient gels (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and blotted with anti-ABCR monoclonal antibody (Rim3F4; a gift of Robert S. Molday, University of British Columbia, Vancouver, BC, Canada) and anti-calnexin rabbit polyclonal antibody (SPA-860; StressGen, Victoria, British Columbia, Canada).

(α-32P)-8-azido-ATP (ICN, Irvine, CA) was used to label membrane proteins, as described.12-14 Under dim red light, (α-32P)-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 MgCl2). Membrane proteins of 2, 4, or 8 µg were diluted to 8 µl in buffer I, and an equal volume of (α-32P)-8-azido-ATP was added. The reactions were allowed to incubate at room temperature for 5 minutes and then irradiated with a handheld 302-nm UV light for 5 minutes at 10 cm. (α-32P)-8-azido-ATP-labeled membranes were separated by SDS-PAGE on 4% to 15% gradient gels, transferred to PVDF membranes, autoradiographed, and blotted with Rim3F4 and SPA-860 antibodies.

For dot blot analysis, 2 µg total RNA from each transfection was spotted onto a nylon membrane (Oncor, Gaithersburg, MD) with a blot transfer apparatus (Bio-Dot; Bio-Rad). The membranes were hybridized with probes to human β-actin or a 1649-bp EcoRI restriction fragment from the ABCR cDNA corresponding to exons 7-16, washed, and autoradiographed.

Films of the membranes from Western, RNA, and ATP labeling experiments were scanned and analyzed with blot analysis software (UN-SCAN IT; Silk Scientific Inc., Orem, UT).

RESULTS

Clinical Summary

Subject AR682-03 first experienced blurred vision at age 15 years. Ophthalmoscopic examination showed a bull’s eye depigmentation of the fovea and a wreath of perifoveal flecks in each eye (Fig. 1A). Fluorescein angiography revealed a dark choroid, and STGD1 was diagnosed at age 17. An ERG showed slightly delayed implicit times but both scotopic and photopic amplitudes were normal. Her maternal grandmother and great aunt were reported to be affected with RP. The paternal grandmother and great aunt were reported to be affected with RP. The paternal grandmother, AR682-04, experienced both night blindness at age 10 years and progressive loss of visual acuity and peripheral fields. At age 53 years, her visual acuity was hand motions only at 6 ft. Her ophthalmic examination documented posterior cortical cataracts, optic atrophy, attenuated retinal vasculature, and scattered bone spicule formation in each eye (Fig. 1B). Her older sister, AR682-05, reported that similar progressive loss of both dim light and peripheral vision had been present since the first decade of life. By age 73, visual acuity was light perception only in each eye, and the retinal examination documented advanced extensive pigmented retinopathy with optic atrophy and dense diffuse bone spicule formations in each eye; she has had diagnosed RP for decades.

ABCR Sequence Results

Complete sequencing of the ABCR gene in STGD1-affected individual AR682-03 and RP-affected individual AR682-04 iden-
null ABCR mutations in STGD and RP

Null ABCR Mutations in STGD and RP

Polymorphisms identified in

subjects AR682-03 and -04 are shown in Table 1. RP-affected individuals (Fig. 2). Polymorphisms identified in

econtaining two missense alterations) discovered in these two families. None of the control individuals (192 chromosomes) carried these alterations, which suggests that these are disease-associated and not benign variants.

Biochemical Analysis of Recombinant ABCR

Plasmid constructs encoding recombinant ABCR proteins bearing the missense mutations V767D, W1408R, and R1640W, and the complex allele [W1408R; R1640W] were transiently transfected into HEK 293T cells. At 36 to 42 hours after transfection, RNA and protein were extracted and analyzed for effects of these pathogenic mutations on protein expression and ATP-binding. All constructs were transfected at least three times, and results were consistent between each iteration of the experiments.

RNA was analyzed by dot blot and hybridization. Total RNA (2 μg) was spotted in quadruplicate onto a nylon membrane, and the membrane was cut in half and hybridized with either a β-actin or ABCR probe. The ratio of ABCR to β-actin signal was used as a measure of transfection efficiency to normalize Western blot analysis. This analysis revealed no significant differences of the disease-associated point mutations on ABCR mRNA expression (Fig. 3).

Western blot analysis of proteins from transfected 293T cells revealed significant defects of the RP-associated ABCR mutations. Western blot analysis of proteins from cells transfected with the V767D or [W1408R; R1640W] mutation-bearing constructs showed very little, if any, protein (Fig. 4). In contrast, proteins bearing the W1408R or R1640W mutations appeared to have mild or moderate defects in expression or stability (Fig. 4). ATP labeling of membranes from transfected cells showed results similar to those of the Western blot analysis. Recombinant ABCR bearing the V767D or [W1408R; R1640W] mutations showed no labeling with azido-ATP, even when fivefold total protein was used in the assay (Fig. 4 and data not shown). The recombinant protein bearing the R1640W mutation had a moderate effect on ATP labeling, with approximately 55% of wild-type activity. As reported by Sun et al., the mutation W1408R had a moderate effect on ATP labeling. Normalized ATP labeling values showed approximately 75% of wild-type labeling for this mutant protein (Fig. 4).

ATP binding to ABCR was tested in the absence of calcium. As reported by Sun et al., this mutation had a moderate effect on ATP binding, with approximately 55% of wild-type activity. As reported by Sun et al., the mutation W1408R had a moderate effect on ATP labeling. Normalized ATP labeling values showed approximately 75% of wild-type labeling for this mutant protein (Fig. 4).

Figure 2: Pedigree AR682 segregates ABCR mutations with both Stargardt disease and RP. Filled circle: Stargardt-affected individual; cross-hatched symbols: RP-affected individuals. Individual subject numbers are shown below each symbol. ABCR genotypes are abbreviated below the individual numbers, with the key to the abbreviations shown at lower left. For brevity, the mutation 2588G —→ C was denoted G863A.

Figure 3: Dot blot analysis of RNA from transfected HEK 293T cells. Dot blot hybridization results with ABCR and β-actin probes are shown for RNA samples from transiently transfected 293T cells. Constructs are indicated above each blot. Transfections were performed multiple times; results from each transfection are shown. HEK 293T indicates untransfected cells that served as a negative control. Note that each mutant construct expressed abundant ABCR mRNA.

Table 1. ABCR Alterations in Patients with Stargardt Disease and Retinitis Pigmentosa

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide</th>
<th>Amino Acid</th>
<th>AR682-03</th>
<th>AR682-04</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>302+26</td>
<td>A/A</td>
<td>A/G</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1268G → A</td>
<td>H423R</td>
<td>A/A</td>
<td>A/G</td>
</tr>
<tr>
<td>1356+11delG</td>
<td>6G/6G</td>
<td>G863A</td>
<td>G/G</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2588G → C</td>
<td>A</td>
<td>T/T</td>
<td>T/A</td>
</tr>
<tr>
<td>17</td>
<td>2300T → A</td>
<td>V767D</td>
<td>T/T</td>
<td>T/A</td>
</tr>
<tr>
<td>19</td>
<td>2828G → A</td>
<td>R943Q</td>
<td>G/A</td>
<td>G/G</td>
</tr>
<tr>
<td>24</td>
<td>3523-30</td>
<td>A/T</td>
<td>A/T</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>4222T → C</td>
<td>W1408R</td>
<td>C/T</td>
<td>C/T</td>
</tr>
<tr>
<td>33</td>
<td>4918C → T</td>
<td>R1640W</td>
<td>C/T</td>
<td>C/T</td>
</tr>
<tr>
<td>55</td>
<td>5605A → T</td>
<td>N1868I</td>
<td>A/T</td>
<td>A/A</td>
</tr>
<tr>
<td>56</td>
<td>5682G → C</td>
<td>L1894L</td>
<td>G/C</td>
<td>G/G</td>
</tr>
</tbody>
</table>

Mutations are indicated in bold.
Among our subjects, none harbored two null ABCR alleles, suggesting that all subjects with STGD1 retain some ABCR activity. Three unrelated pedigrees segregated ABCR mutations with recessive RP19. In each of these three families, the RP-associated mutations have been truncating or splice mutations, consistent with the hypothesis that in individuals without ABCR activity, early-onset, severe, and rapidly progressive retinal dystrophy develops, with a clinical phenotype similar to classic RP and entirely distinct from STGD.

Pedigree AR682 manifests both STGD1 and RP. We performed extensive sequence analyses of the ABCR gene in one STGD1 proband and one RP-affected individual from this pedigree. This search yielded three different mutant ABCR alleles (Fig. 2).

Pedigree AR682 segregated missense ABCR mutations with both STGD1 and RP. The 2588G→C alteration in STGD1 patient AR682-03 has been observed previously in 26 unrelated patients with STGD1 and has been classified as a mild mutant allele based on its association with later onset disease and its pairing with presumed severe alleles in patients with STGD1. In addition, we observed the polymorphism 2828G→A in cis to the 2588G→C alteration, consistent with linkage disequilibrium between these two alterations, as reported previously. The effects of the mutation 2588G→C have been studied by Sun et al., who report a moderate reduction in expression of the G863A mutant protein and a modest reduction in ATP-binding for the G863del variant of the 2588G→C mutation.

In individual AR682-03, the combination of the 2588G→C allele with the complex allele [W1408R; R1640W] resulted in STGD1 with onset of visual symptoms at age 15 years, consistent with classification of the [W1408R; R1640W] allele as moderate to severe. RP-affected individuals AR682-04 and -05 carry the complex allele [W1408R; R1640W] in trans to the missense allele V767D. Both have advanced RP at ages 71 and 73 years, each with a diagnosis of RP for over five decades.

The three previously reported pedigrees with ABCR-associated RP had progressive retinal deterioration with onset of night blindness before age 10, progressive rod and cone dystrophy including loss of central vision, and pigment epithelial and choroidal atrophy. For each of the RP-affected individuals presented in this study, symptomatic nyctalopia occurred in the late first or early second decade and progressed to loss of functional central acuity (e.g., loss of automotive license) by the fifth decade. The retinal examinations in these adult subjects showed advanced RP.

ATP labeling and Western blot analysis of recombinant ABCR shows that these mutant proteins are not efficiently expressed in our transient transfection system, despite expression of substantial amounts of mRNA (Figs. 3, 4). Based on our new observations, we predict that both RP-associated alleles V767D and [W1408R; R1640W] represent severe mutations that result in little or no ABCR activity. Either these mutations cause misfolding of ABCR, or the nascent protein is unstable and quickly degraded, in that little or no protein could be detected by Western blot analysis and ATP labeling. In support of the misfolding hypothesis, the mutation V767D is predicted to lie within a transmembrane region and may disrupt proper folding of the nascent protein. However, the alterations W1408R and R1640W are each predicted to affect the first intradiscal loop, which has no known function.

Of interest, the mutation V767D was reported in combination with the mutation 250delCAA (a frameshift mutation that is a presumed null allele) in a patient with STGD1 with onset at age 8 years. That this patient (now 24 years old) has an ABCR genotype predicted to be equal to or more severe than that of our patient with advanced RP suggests a potential role for environmental or modifier gene effects.

The effects of the W1408R mutation on ABCR expression and ATP binding are consistent with the classification of this as a mild to moderate mutation. Our results confirm and refine those reported by Sun et al., who show that this mutation has approximately 75% of wild-type expression and ATP-binding activity. Analysis of the R1640W mutation revealed a reduction in both expression (~65% wild-type) and ATP-binding capacity (~60% wild-type). Furthermore, our analysis of the mutations W1408R and R1640W alone and in combination suggest that the null effects of the complex allele [W1408R; R1640W] are due to a combination of the two alterations, and are more severe than either mutation alone (Fig. 4). This represents an important finding, because complex alleles have been reported in 7% of our unselected cohort of 150 STGD-affected families, as well as in a large number of other reported patients with STGD.

Our analysis and identification of missense ABCR mutations in a family that segregates both STGD and RP is novel and interesting. Based solely on the mutation data and model of ABCR activity and its relation to pathogenesis, we hypothe-
sized that the missense mutation identified in patients with RP would have severe functional consequences. Biochemical analysis of recombinant ABCR bearing these mutations confirmed that the RP-associated missense mutations are null, and further demonstrated that the effects of the complex allele W1408R; R1640W are more severe than a simple additive effect of the two constituent mutations. Our findings further support a model in which disease severity is inversely correlated with ABCR activity and extend the model to include missense mutations associated with RP.

Acknowledgments

The authors thank family AR682 and their attending physicians, Robert D. Gourley, MD, Ronald M. Kingsley, MD, Alan L. Maberley, MD, Stephen R. Martin, MD, Leslie W. Nesmith, MD, Raymond E. Townsend, MD, and Mark J. Weiss, MD, for their willing and continued cooperation in these studies; Jeremy Nathans, MD, PhD, and Robert S. Molday, PhD, for reagents and advice; and Theodore Wensel, PhD, for abundant assistance with the biochemical analysis of ABCR.

References