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ARTICLE

Autosomal recessive retinitis pigmentosa and cone–rod dystrophy caused by splice site mutations in the Stargardt's disease gene *ABCR*

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Ophthalmological and molecular genetic studies were performed in a consanguineous family with individuals showing either retinitis pigmentosa (RP) or cone–rod dystrophy (CRD). Assuming pseudodominant (recessive) inheritance of allelic defects, linkage analysis positioned the causal gene at 1p21–p13 (lod score 4.22), a genomic segment known to harbor the *ABCR* gene involved in Stargardt's disease (STGD) and age-related macular degeneration (AMD). We completed the exon–intron structure of the *ABCR* gene and detected a severe homozygous 5′ splice site mutation, IVS30+1G→T, in the four RP patients. The five CRD patients in this family are compound heterozygotes for the IVS30+1G→T mutation and a 5′ splice site mutation in intron 40 (IVS40+5G→A). Both splice site mutations were found heterozygously in two unrelated STGD patients, but not in 100 control individuals. In these patients the second mutation was either a missense mutation or unknown. Since thus far no STGD patients have been reported to carry two *ABCR* null alleles and taking into account that the RP phenotype is more severe than the STGD phenotype, we hypothesize that the intron 30 splice site mutation represents a true null allele. Since the intron 30 mutation is found heterozygously in the CRD patients, the IVS40+5G→A mutation probably renders the exon 40 5′ splice site partially functional. These results show that mutations in the *ABCR* gene not only result in STGD and AMD, but can also cause autosomal recessive RP and CRD. Since the heterozygote frequency for *ABCR* mutations is estimated at 0.02, mutations in *ABCR* might be an important cause of autosomal recessive and sporadic forms of RP and CRD.

INTRODUCTION

Monogenic chorioretinal degenerations display exceptional clinical and genetic heterogeneity (1,2). Their clinical classification is partly based on the location of lesions in the retina early in the disease. Progressive peripheral vision loss is seen in, for example, classical retinitis pigmentosa (RP), the Usher syndromes, choroideremia, Bardet–Biedl syndrome and Kearns–Sayre syndrome. In contrast, central vision loss due to macular dystrophy is observed in, for example, Stargardt disease, central areolar choroidal dystrophy, Sorsby's fundus dystrophy, cone and cone–rod dystrophies and Best's vitelliform macular dystrophy.

Over the last 7 years molecular genetic studies have revealed the underlying gene defects in many of these disorders. Classical forms of dominant RP can be due to mutations in the rhodopsin or peripherin/*RDS* genes (3–5). Autosomal recessive RP can be caused by mutations in the rhodopsin gene (6), the gene encoding the α- or β-subunit of rod cGMP phosphodiesterase (7,8) or the gene encoding the α-subunit of the rod cGMP-gated channel (9). Mutations in the *RPGR* gene are associated with X-linked RP (10,11). The peripherin/*RDS* gene has also been involved in a myriad of dominant macular dystrophies, including cone–rod dystrophy (CRD), macula dystrophy, pattern dystrophy and central areolar choroidal dystrophy (12–16). Sorsby's fundus

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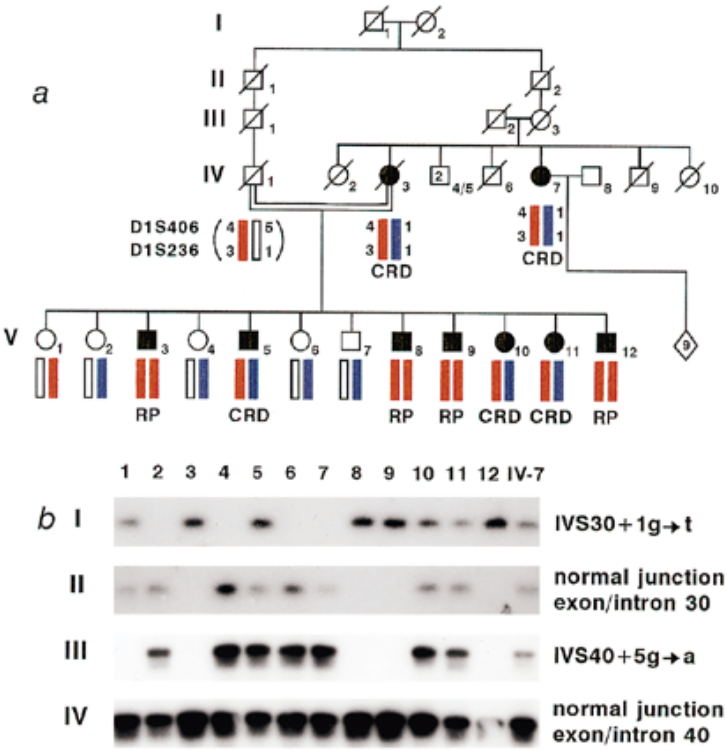

Figure 1. Linkage analysis in the 1p21 region and allele-specific oligonucleotide hybridization analysis of the RP/CRD family. (**a**) The brackets flanking the D1S406 and D1S236 haplotypes for individual IV-1 indicate that they were deduced from the haplotypes of his children. The diamond symbol denotes nine healthy children of IV-7 and IV-8. (**b**) ASO hybridization results using the mutant (panel I) and wild-type (panel II) primers designed from the exon 30–intron 30 junction sequences. ASO hybridization of mutant (panel III) and normal (panel IV) primers designed from the exon 40–intron 40 junction sequences.

dystrophy is caused by mutations in the tissue inhibitor of the metalloproteinases type 3 (*TIMP3*) gene (17). Mutations in a gene encoding a novel retina-specific ATP binding cassette (ABCR) protein are associated with recessive Stargardt's disease (STGD)/fundus flavimaculatus (FFM) and age-related macular degeneration (AMD) (18,19). Finally, a novel X-linked gene (*XLRS1*) was implicated in X-linked juvenile retinoschisis (20) and a gene for a photoreceptor-specific transcription factor, *CRX*, was mutated in autosomal dominant cone–rod dystrophy (21).

In this study we report on a consanguineous family which 27 years ago was shown to suffer from a 'centroperipheral' tapetoretinal dystrophy (22). A re-evaluation of the clinical features in patients of this family revealed two different phenotypes, a cone–rod dystrophy in some and retinitis pigmentosa in other patients. Linkage analysis showed co-segregation of the disease phenotypes with alleles of markers from the 1p21–p13 region flanking the STGD gene *ABCR*. Elucidation of the complete exon–intron structure and sequence analysis of all exons of the *ABCR* gene revealed a homozygous intron 30 splice site mutation in the RP patients and compound heterozygosity for the same intron 30 mutation and an intron 40 splice site mutation in the CRD patients.

RESULTS

Clinical evaluation

Re-evaluation of a consanguineous family which 27 years ago was shown to suffer from a 'centroperipheral' tapetoretinal dystrophy (22) showed that four patients were affected with retinitis pigmentosa (RP) and five with cone–rod dystrophy (CRD) (Fig. 1a). Individuals IV-1 and IV-3 are related through great-grandparents, suggesting autosomal recessive (pseudodominant) inheritance. In agreement with recessive inheritance, the parents of IV-3 and IV-7 are unaffected. Fundus examination and electroretinography of patients IV-3, IV-7, V-5, V-10 and V-11 shows a dystrophy in the cone and rod systems. The onset of visual loss occurred between the ages of 12 and 15. In patients V-5 and V-11 visual field testing at the ages of 26 and 44 respectively showed a central scotoma and constriction of the peripheral visual field. The visual acuity in these patients was reduced to finger counting at 1 m. In patients V-5 (age 54; Fig. 2A), V-10 and V-11 (age 44; Fig. 2B) funduscopy showed marked chorioretinal atrophy of the macula. The optic disks, retinal vessels and periphery of the retina were normal. In their mother (IV-3) and maternal aunt (IV-7) the area of chorioretinal atrophy was much larger at ages 72 and 82 respectively. The optic disks in the latter two patients showed some pallor. Together, this phenotype closely resembles CRD. The phenotype of the CRD patients differs from STGD in that the CRD patients from our family have never shown flecks and show no 'dark choroid' upon fluorescein angiography (23,24). At later stages STGD patients may show a subnormal b-wave amplitude in electroretinograms (ERG), whereas the CRD patients in this family show a severely diminished b-wave amplitude typical of late stage CRD patients (25).

Patients V-3, V-8, V-9 and V-12 show a classical retinitis pigmentosa (RP) picture. Their first visual complaints started around the age of 6; all of them reported night blindness as one of the first symptoms. Their visual acuity ranged from light perception to hand movements at 1 m. The ERG responses of the

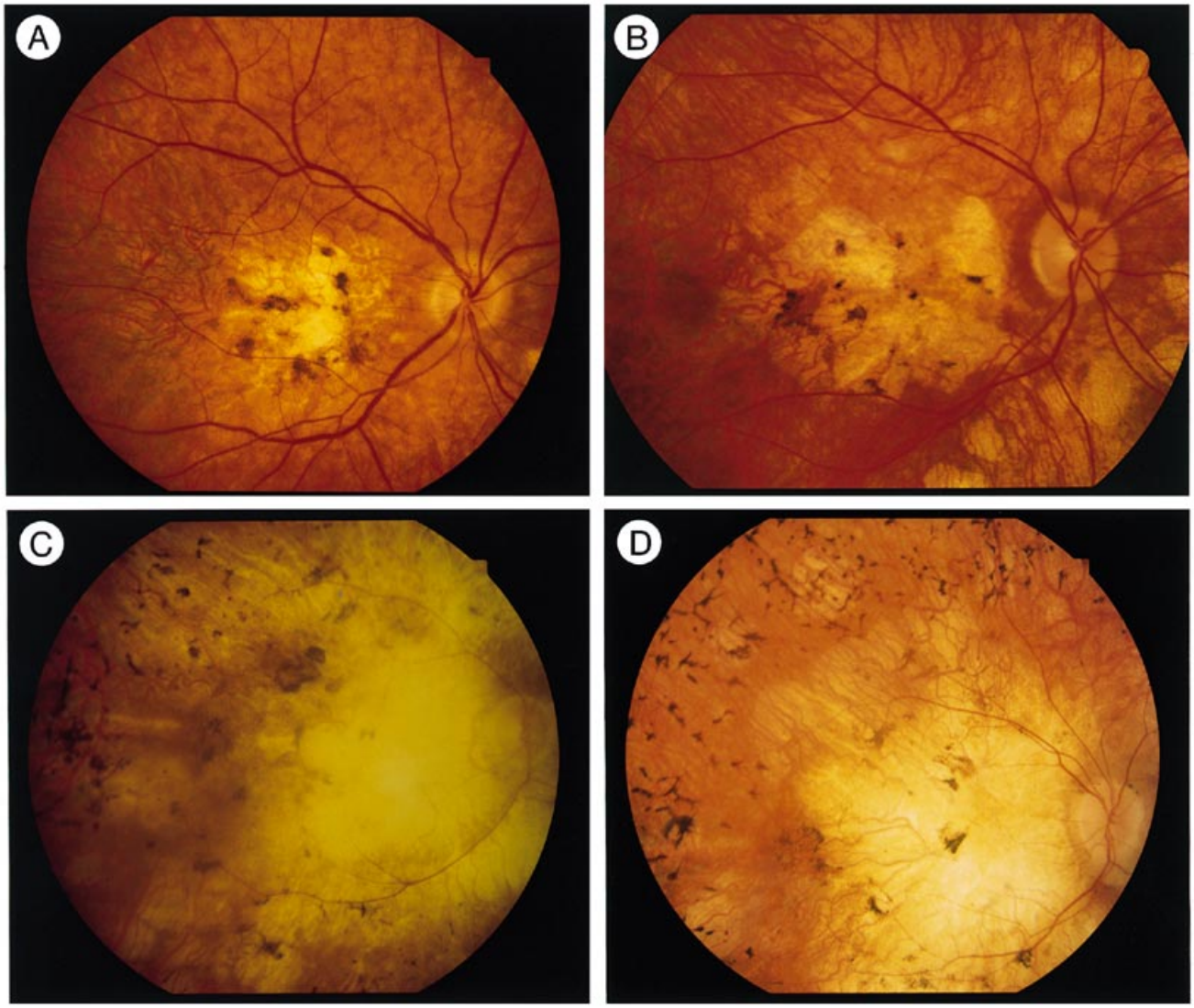

Figure 2. Fundus appearance of affected individuals. CRD patients V-5 (**A**) and V-11 (**B**) show chorioretinal degeneration predominantly in the posterior pole; the (mid)periphery, retinal vessels and optic disks are normal. RP patients V-3 (**C**) and V-12 (**D**) show extensive chorioretinal dystrophy, attenuated retinal vessels, bone spicules in the (mid)periphery and a waxy pallor of the optic disks. The fundus picture in (B) was taken at a higher magnification compared with (A), (C) and (D).

rods and cones were hardly detectable or extinguished. These sibs had marked chorioretinal atrophy of the posterior pole, a 'waxy' pallor of the optic disk, attenuated retinal vessels and bone spicules in the (mid)periphery of the retina. These findings are exemplified by the fundus pictures of patient V-3 (age 57; Fig. 2C) and patient V-12 (age 41; Fig. 2D). A more detailed clinical description of this family will be published elsewhere.

Linkage and haplotype analysis

As some of the patients showed lesions in the central part of the retina, we tested the possibility that the molecular defects in this family were located in or near the critical region for STGD. Assuming autosomal recessive inheritance of allelic defects, the STGD flanking markers D1S406 and D1S236 (26,27) showed no recombinations with the disease phenotype(s), with a maximal lod score of 4.22 for marker D1S406. All affected sibs in generation V inherited the same chromosomal segment from their healthy father (red haplotype; Fig. 1a). From their affected (CRD) mother the RP patients inherited the red haplotype and the CRD patients the blue haplotype (Fig. 1a).

We extended the haplotypes to determine whether our findings were consistent with homozygosity by descent for the RP patients and to establish the critical region for the gene defects. All RP patients showed homozygosity for eight highly informative markers from 1p21, i.e. D1S198, D1S311, D1S167, D1S406, D1S236, D1S497, D1S206 and D1S495 (data not shown). In the D1S198–D1S250 region crossover events were exclusively found in the CRD patients (Fig. 3). The telomeric demarcation of the critical region for RP/CRD is based on a recombination

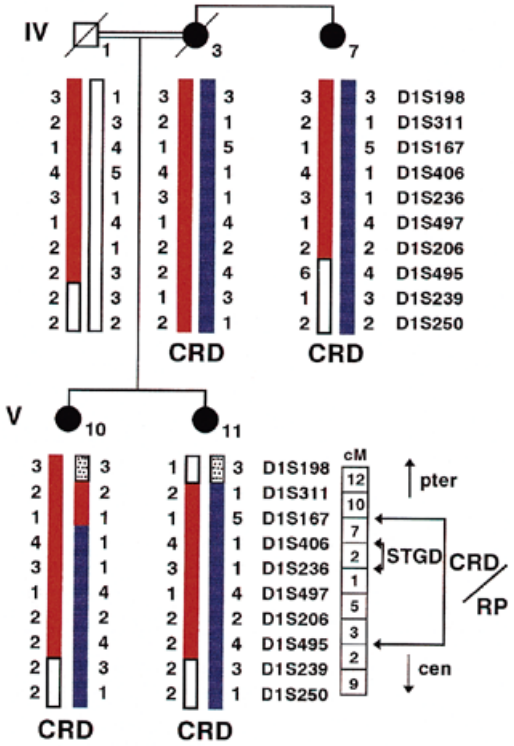

Figure 3. Illustration of the recombinant chromosomes delineating the CRD/RP region at 1p21–p13. The red parts of the bars contain the allele which, when present homozygously, results in RP. The blue segments contain a disease allele which, when present in combination with the 'RP allele', results in CRD. The genetic distances between the markers were deduced from other studies (26,43) and are given in cM. The stippled segments denote non-informativity of markers. The non-informativity of D1S206 in individuals V-10 and V-11 is not indicated since a double crossover within 8 cM is required to postulate inheritance of the maternal D1S206 allele from the red haplotype. The haplotype of individual IV-1 was deduced from his children's haplotypes (not shown).

observed in V-10. Assuming that V-10 suffers from CRD due to compound heterozygosity for gene defects in this region, the CRD/RP critical region is situated proximal to D1S167. The proximal boundary for the location of the gene defects is based on a crossover between D1S206 and D1S495 observed in IV-7 (Fig. 3). The D1S167–D1S495 region encompasses ~18 cM and spans the STGD critical region, which was situated between D1S406 and D1S236 (Fig. 3).

The *RPE65* gene, recently implicated in some families with Leber's congenital amaurosis (28) and severe autosomal recessive childhood onset retinal dystrophy (29), was positioned outside the 18 cM interval by Anderson and co-workers (26). The human cone transducin α-subunit gene (*GNAT2*) maps in or near the 18 cM region (30). A previously reported polymorphic *Dsa*I restriction site in the *GNAT2* gene (31) was not informative in our family (data not shown). Therefore, we performed single-strand conformation (SSC) analysis employing DNA from all family members and sequence analysis using DNA of patients IV-7 and V-3. No SSC variants or mutations were found in the open reading frame of the *GNAT2* gene.

Mutation analysis of the *ABCR* gene

While these studies were in progress Allikmets and co-workers published the cDNA and part of the exon–intron structure of the

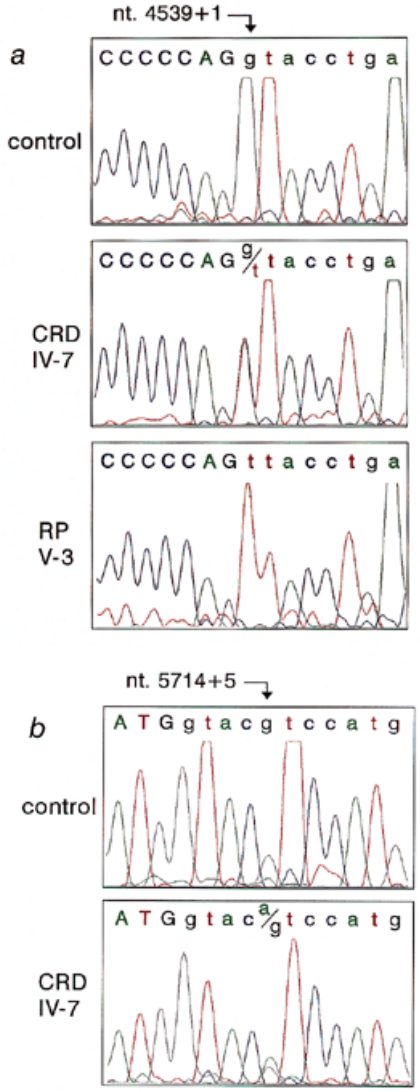

Figure 4. Normal and mutant DNA sequences of the 5′ splice sites of introns 30 and 40. (**a**) Exon 30–intron 30 junction sequence in a control individual (upper panel), a heterozygous CRD patient (IV-7, middle panel) and a homozygous RP patient (V-3, lower panel). (**b**) Exon 40–intron 40 junction sequence in a control individual (upper panel) and a heterozygous CRD patient (IV-7, lower panel).

STGD gene *ABCR* (18). Sequence analysis of the 21 exons for which flanking oligonucleotides were described, i.e. exons 15–25, 27, 33–36, 43–46 and 48, did not reveal causative mutations in patients IV-7 and V-3. By performing exon–exon PCR, we therefore completed the exon–intron structure of the *ABCR* gene, which was shown to consist of 50 exons (32). Sequence analysis of the remaining 29 *ABCR* exons in RP patient V-3 and CRD patient IV-7 revealed 5′ splice site mutations downstream of exons 30 and 40. In RP patient V-3 the G nucleotide at position 4539+1 was mutated homozygously to a T, thereby inactivating the 5′ splice site of intron 30 (Fig. 4a). CRD patient IV-7 is heterozygous for this mutation (Fig. 4a). Hybridization of normal and mutant allele-specific oligonucleotides (ASOs) spanning the exon 30–intron 30 junction to amplified genomic DNA from relevant family members shows that all RP patients are homozygous for the IVS30+1G→T mutation; the CRD patients and one healthy

individual (V-1) from this family are heterozygous (Fig. 1b, panel I). As expected, the wild-type ASO (IVS30-5W) hybridized to the DNA of all individuals except the RP patients (Fig. 1b, panel II).

In the 5′ splice site of intron 40 of the *ABCR* gene in CRD patient IV-7 we encountered a heterozygous mutation, 5714+5G→A (Fig. 4b). Except for this mutation and the IVS30+1G→T alteration, no other mutations were found in the *ABCR* gene of CRD patient IV-7. All CRD patients and healthy individuals V-2, V-4, V-6 and V-7 carry the same mutation in one *ABCR* allele (Fig. 1b, panel III). The wild-type ASO (IVS40-5W) hybridized to all DNAs tested, since neither of the individuals was homozygous for the IVS40+5G→A mutation (Fig. 1b, panel IV).

IVS30 and IVS40 splice site mutation analyses in STGD patients

Using ASO hybridization, the IVS30+1G→T mutation was also found heterozygously in two of 59 unrelated STGD patients (7560 and 7727; Table 1), but not in 100 control individuals (data not shown). STGD patient 7560 in addition carries a G→C transversion at position 2588 in exon 17, resulting in a Gly863Ala mutation in the predicted ABCR protein (Table 1). The Gly863Ala mutation was found in another 20 of a total of 58 STGD patients, but not in 100 control individuals. The Gly863Ala mutation thereby is one of the most frequently observed mutations in our STGD patient cohort. Analysis of ~50% of the *ABCR* open reading frame (ORF) has not yet revealed the mutation in the second allele of patient 7727.

The IVS40+5G→A mutation was found heterozygously in two unrelated STGD patients (8272 and 8439), but not in the DNA of 100 control individuals. STGD patient 8439 carries a C→T transition at nucleotide position 3113, resulting in an Ala1038Val mutation in the predicted ABCR protein (Table 1). The same mutation was found in five of 59 STGD patients investigated by us and in 15 of 150 STGD patients studied by Allikmets, but not in a total of 200 control individuals (our own observation and R.Allikmets, personal communication). This mutation was previously erroneously designated Ala1028Val (18; R.Allikmets, personal communication). *ABCR* mutation analysis in ~50% of the ORF has not yet revealed the second mutation in STGD patient 8272.

DISCUSSION

In families with recessively inherited gene defects affected sibs are either compound heterozygotes, when their parents are unrelated, or homozygotes, if their parents are consanguineous. In both cases they will inherit the same set of gene defects. In families showing pseudodominant inheritance sibs can inherit different sets of mutations, but phenotypical differences will be minimal because generally both recessive gene defects are null alleles. In this study we have analyzed a pseudodominant family in which affected sibs do show different clinical features, i.e. they either show a classical RP phenotype or CRD. Linkage and haplotype analysis showed co-segregation of the gene defects with the STGD critical region and mutation analysis of the *ABCR* gene revealed 5′ splice site mutations in introns 30 and 40. Interestingly, the RP patients show homozygosity for the IVS30+1G→T mutations, whereas all CRD patients are compound heterozygotes for the IVS30+1G→T and IVS40+5G→A mutations. Although our findings allow genotype–phenotype comparisons, a faithful prediction of the effect of the IVS30 and IVS40 mutations on the *ABCR* mRNA can only be made upon detailed RNA splicing analyses. Thus far, nested RT-PCR studies using RNA isolated from Epstein–Barr virus-transformed cell lines from RP and CRD patients did not give reproducible results, which is not too surprising given the retina-specific expression of the *ABCR* mRNA (18,34).

Allikmets and co-workers, as published previously and as described in a ‘correction’ (18), identified two different *ABCR* cDNAs. In one variant there is a 114 bp insertion after nucleotide position 4352, which is explained by alternative or aberrant splicing of an additional exon. After careful inspection of the cDNA sequence we identified a cryptic 3′ splice site at position 4467 in exon 30, which, according to the splice site weighting method of Shapiro and Senapathy (33), has a ‘splice potential score’ of 90.6, compared with the 83.0 of the 3′ splice site at position 4353. If the cryptic splice site is used (Fig. 5, variant II), 114 nt of exon 30 are skipped, leading to a 38 amino acid deletion in the predicted ABCR protein. Whether the alternative use of the 3′ splice sites of exon 30 has any functional relevance remains to be investigated. Inactivation of a 5′ splice site in general results in skipping of the preceding exon (35), which in the case of the IVS30+1G→T mutation would lead to a reading frame shift in exon 31 and the absence of transmembrane domains VII–XII, as well as the second ATP binding cassette and the Walker A and B motifs of the ABCR protein (Fig. 5, splicing variant A). Thirty nucleotides downstream of the mutated 5′ splice site of exon 30 there is a cryptic 5′ splice site (AGgtctgc) which has a splice potential score of 72.8, which is comparable with the score of the normally used splice site at position 4539 (score 74.3; Fig. 5). If the cryptic 5′ splice site is used there will be a 10 amino acid insertion after amino acid position 1513 (in ref. 18, amino acid position 1475) of the predicted ABCR protein (Fig. 5, splice variant B). The IVS30+1G→T mutation was also observed in two STGD patients, one of which carried a Gly863Ala mutation in the second allele.

Table 1. Genotype–phenotype comparison for the RP, CRD, STGD/FFM and AMD patients and a hypothetical correlation between ABCR activity and the observed phenotypes

Patient	Phenotype	*ABCR* allele 1	*ABCR* allele 2	ABCR activity
7023 (V-3)	RP	IVS30+1G→T	IVS30+1G→T	–
7028 (IV-7)	CRD	IVS30+1G→T	IVS40+5G→A	+/–
7560	STGD	IVS30+1G→T	Gly863Ala	+
7727	STGD	IVS30+1G→T	Unknown	+
8439	STGD	IVS40+5G→A	Ala1038Val	+
8272	STGD/FFM	IVS40+5G→A	Unknown	+
	AMD	Missense or null mutation (19)		++
	Normal			++++

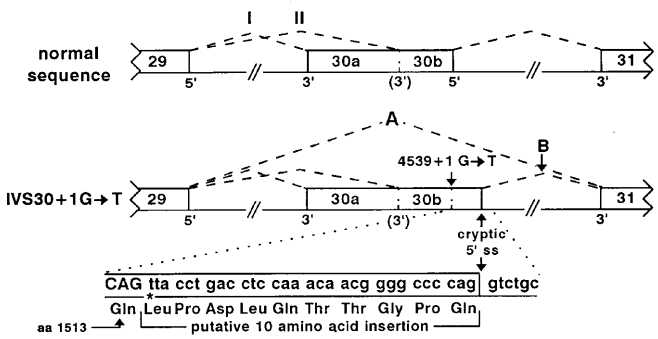

Figure 5. Schematic representation of *ABCR* RNA splicing variants around exon 30. In the normal *ABCR* sequence (upper drawing) two splice variants have been observed (18). Splice variant II is caused by a cryptic 3′ splice site at position 4467. Due to the IVS30+1G→T mutation (lower drawing), exon 30 might be skipped (variant A) or the splicing machinery utilizes a cryptic 5′ splice site 30 nt downstream of the normally used 5′ splice site at position 4539 (variant B). In the latter variant 10 aberrant amino acids will be inserted into the ABCR protein. The asterisk indicates the mutated nucleotide.

The IVS40+5G→A splice site mutation lowers the splice potential score from 80.7 to 66.2, which suggests that splicing might still be possible at this site in a low percentage of *ABCR* transcripts. The pathological nature of this mutation is underlined by its heterozygous occurrence in two of our 59 STGD patients (Table 1) and in three of 100 STGD patients investigated by Allikmets and co-workers (R.Allikmets, personal communication). In contrast to the 5′ splice site of exon 30, there is no potential cryptic 5′ splice site up to 180 bp downstream of exon 40 and skipping of exon 40 would lead to a reading frame shift in exon 41. The result would be deletion of the XII transmembrane region, the second ATP binding cassette and the Walker A and B motifs of the ABCR protein.

Given the more severe clinical picture in RP patients compared with CRD and STGD patients, it can be deduced that homozygosity for the IVS30+1G→T mutation, associated with an RP phenotype, is more detrimental to ABCR activity than compound heterozygosity for the IVS30+1G→T and IVS40+5G→A mutations, which results in a CRD phenotype, or compound heterozygosity for the IVS30+1G→T and Gly863Ala mutations, which results in an STGD phenotype. This conclusion is corroborated by the fact that thus far we and others have not yet identified two *ABCR* null mutations in STGD patients. Therefore, we hypothesize that every combination of two null alleles in the *ABCR* gene results in autosomal recessive RP and that a combination of an *ABCR* null allele with a mutation yielding residual ABCR function, as for example the IVS40+5G→A mutation, can result in a CRD picture.

Another family with RP (RP19) was recently mapped in the vicinity of the *ABCR* gene (36), suggesting that *ABCR* mutations are also the cause of the atypical RP described in this family. The fact that RP and CRD can be caused by mutations in the same gene is not without precedent. Previously mutations in the peripherin/*RDS* gene were associated with a myriad of different phenotypes, among which were autosomal dominant forms of RP and CRD (4,12,14–16). Although the molecular basis for the variable phenotypes associated with *ABCR* mutations remains to be elucidated, identification of RP-specific mutations is in agreement with rod-specific expression of the *ABCR* gene (18,34). The *ABCR* gene is the fifth gene implicated in autosomal recessive RP and the first gene found to be involved in an autosomal recessive form of CRD. The heterozygote frequency for *ABCR* gene mutations is estimated at 0.02. Since we observe *ABCR* null mutations in ~20% of the STGD alleles, mutations in the *ABCR* gene can be predicted to cause RP in ~1/250 000 individuals. Our studies show that depending on the combination and severity of mutations in the *ABCR* gene, the phenotypic outcome can be a continuum ranging from RP at one end of the spectrum to AMD at the other end.

MATERIALS AND METHODS

Ophthalmological examinations

The CRD/RP family was initially examined in 1971 (22). In 1995 two sibs of generation IV (IV-3 and IV-7) and all sibs of generation V were re-examined. In these subjects standard ophthalmological examination and fundus photography was carried out and blood samples were obtained. Electrophysiological examinations were performed in selected cases. STGD patients 7560 and 7727 and STGD/FFM patient 8272 are from The Netherlands and were clinically examined by A.J.L.G.P. and N.T. STGD patient 8439 is from Germany and was clinically examined by K.R. and A.B.

Linkage and haplotype analysis

DNA was extracted from leukocytes as described previously (37). Microsatellite DNA markers of the 1p21–p13 region were used to genotype patients IV-3 and IV-7 and all children of individual IV-3. DNA amplifications were performed in 96-well trays in an MJ Research thermocycler, with primer pairs as published for the markers (from 1pter to cen) D1S198 (AFM074za5), D1S311 (C81), D1S167 (Utsw 1346; 38), D1S406 (UT2069), D1S236 (AFM205ta11), D1S497 (AFM331vb1), D1S206 (AFM113xf6), D1S495 (AFM323ya5), D1S239 (AFM205yg3) and D1S250 (AFM240yg1). Amplification was performed over 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 1 min. In addition, an initial denaturation step of 4 min and a final extension step of 6 min were carried out. Two-point linkage analyses were performed with the program LINKAGE version 5.03 (39) and the subroutine Mlink. Allele frequencies determined in Centre d'Etude du Polymorphisme Humain families (40) (GDB) were used for lod score calculations. A penetrance of 100% and a disease allele frequency of 0.001 were used.

Mutation analysis of the human cone transducin α-subunit (*GNAT2*) and *ABCR* genes

SCC analysis and sequencing was carried out for the eight *GNAT2* and the 50 *ABCR* exons using primers and methodology as described elsewhere (18,30,32). For exon 30 we employed primers ABCR30f (5′-GTCAGCAACTTTGAGGCTG-3′) and ABCR30r (5′-TCCCTCTGTGGCAGGCAG-3′); for exon 40 we employed primers ABCR40f (5′-AGCTGGGCGGCTGAAG-3′) and ABCR40r (5′-TGCCCTGAGCTGCCCAC-3′). The ASOs were the following 17mers: IVS30-5W (5′-CCCCCCAGgtacctgac-3′), wild-type exon 30–intron 30 junction; IVS30-5M (5′-CCCCCCAGttacctgac-3′), mutant exon 30–intron 30 junction; IVS40-5W (5′-AATGgtacgtccatgcc-3′), wild-type exon 40–intron 40 junction; IVS40-5M (5′-AATGgtacatccatgcc-3′), mutant exon 40–intron 40 junction. Nucleotide sequence analysis was performed with the dye terminator sequencing kit on an Applied Biosystems automated sequencer as described (41). ASO hybridization and

washing was carried out as described by Shuber and co-workers (42).

ABBREVIATIONS

ABCR, retina-specific ATP binding cassette transporter protein; ASO, allele-specific oligonucleotide; CRD, cone–rod dystrophy; IVS, intervening sequence; RP, retinitis pigmentosa.

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