A homozygous deletion in \textit{RPE65} in a small Sardinian family with autosomal recessive retinal dystrophy

William J. Poehner,1 Maurizio Fossarello,2 Ana Lia Rapoport,1 Tomas S. Aleman,3 Artur V. Cideciyan,2 Samuel G. Jacobson,3 Alan F. Wright,4 Michael Danciger,1,5 Debora B. Farber1,6

1 Jules Stein Eye Institute and 4 Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA; 2 Oculista University, Via Ospedale, Cagliari, Italy; 3 Scheie Eye Institute, University of Pennsylvania, Philadelphia, PA; 4 MRC Human Genetics Unit, Western General Hospital, Crewe Road Edinburgh, Scotland; 5 Department of Biology, Loyola Marymount University, Los Angeles, CA

Purpose: We have been engaged in an ongoing study to screen candidate genes for mutations in small families with various forms of autosomal recessive retinal dystrophy. Here we report the screening of a cohort of 14 families from Sardinia for mutations in the genes encoding the alpha- and beta-subunits of cGMP-phosphodiesterase and \textit{RPE65} (\textit{PDE6A}, \textit{PDE6B}, and \textit{RPE65}).

Methods: Haplotype analysis was performed on each family using simple sequence repeat markers closely flanking or within each of the three gene candidates. For families in which a gene could not be ruled out from segregating with disease, exons of the gene from proband DNAs were screened for mutations by SSCPE (single strand conformation polymorphism electrophoresis). All variants found by SSCPE were sequenced directly.

Results: By haplotype analysis, 6/14, 11/14, and 4/13 families were ruled out for \textit{PDE6A}, \textit{PDE6B}, and \textit{RPE65}, respectively. A few variants were found in the proband DNAs of the remaining families, but only one was significant: a 20 bp deletion in exon 4 of \textit{RPE65}. The deletion co-segregated with disease in one family and caused a frame shift that produces a stop codon downstream. It was absent from the other Sardinian families that we tested, and from Sardinian and North American controls. Results of studies of phenotype in homozygotes and heterozygotes in this Sardinian family are compared with those from a non-Sardinian family recently reported to have the same \textit{RPE65} mutation.

Conclusions: This \textit{RPE65} mutation, which appears to be quite restricted in its occurrence in Sardinia, leads to childhood onset severe retinal dystrophy or Leber congenital amaurosis. Affecteds of the other 13 plus two additional families were diagnosed with arRP. This family lived in an area of Sardinia where none of the others lived suggesting different ancestral origins.

In an ongoing study, we have been screening vision genes for mutations in small nuclear families with an autosomal recessive inheritance of various forms of retinal dystrophy. For each candidate gene, our approach has been to first do haplotype analysis on the members of each family with markers that closely flank or are within the gene. Some families can be ruled out for a gene if the markers clearly show that the gene does not co-segregate with disease. In those families where a gene cannot be ruled out from co-segregating with disease, the proband DNA is screened for mutations in the gene’s exons. The exception to this approach is to do exon-screening directly when the candidate gene is small and can be evaluated in its entirety with just a few amplified fragments. Within our collection of more than 100 small autosomal recessive retinal dystrophy families, there is a cohort of 14 families from the island of Sardinia. In each family, there are two or more affected siblings and neither parent has had disease. This cohort is interesting because Sardinia is one of the most extreme genetic outliers in Europe, and has a high degree of genetic homogeneity among its people [1,2].

To date, at least 17 genes have been associated with either Leber congenital amaurosis (LCA), childhood-onset severe retinal dystrophy (CSRDR) or autosomal recessive retinitis pigmentosa (arRP), and another 7 linked to chromosomal regions with yet undiscovered genes [3]. We report here the screening of three of these genes in the Sardinian cohort: \textit{PDE6A}, \textit{PDE6B}, and \textit{RPE65} encoding the alpha- and beta-subunits of cGMP-phosphodiesterase (cGMP-PDE), and the \textit{RPE65} protein, respectively. Mutations in \textit{PDE6A}, \textit{PDE6B} and \textit{RPE65} have been associated with autosomal recessive retinal dystrophies [4-16].

In the last few years, there have been several reports in the literature of mutations in the \textit{RPE65} gene associated with autosomal recessive retinal degenerations of varying degrees of severity including LCA, CSRDR and arRP [12-16]. This is not surprising since the \textit{RPE65} gene has been shown to encode a highly conserved 61-kDa protein expressed abundantly in the retinal pigment epithelium (RPE) [17-20]. Although the specific role of \textit{RPE65} has not been determined, studies in \textit{Rpe65}+/ mice suggest that it is involved in the re-synthesis of 11-cis-retinal, the chromophore of the visual pigment rhodopsin [21]. Based on a recent study demonstrating recovery of function with an oral retinoid in this \textit{Rpe65}+/ murine model [22], it may become increasingly important to identify patients with retinal degenerations caused by \textit{RPE65} mutations.
After ruling out those families that did not segregate with the three genes studied, the exons of the genes from the DNAs of probands of the remaining families were screened for mutations by SSCP/E. We found one homozygous deletion in RPE65 that cosegregated with disease in one of the families, and no mutations in the other two genes.

**METHODS**

DNA was extracted from blood leukocytes by standard methods and with informed consent of the patients. The DNAs were analyzed for the alleles of the following simple sequence repeat markers: PDEB which is in the PDE6B gene [23,24]; D5S2013 and CSF1R which closely flank the PDE6A gene; and D1S198, D1S219 and D1S2803 for the RPE65 gene. D1S198 and D1S219 closely flank RPE65, and D1S2803 is in a region upstream of the promoter [25]. A fragment of the PDE6A gene was scored in the G3 radiation hybrid panel (Research Genetics, Huntsville, AL) and mapped in the Stanford Human Genome Center Radiation Hybrid database. PDE6A was positioned within a 10-cR8000 region between CSF1R and D5S2013 (10-cR8000 is approximately 300 kb). The RPE65 gene has been placed within a 13-cR8000 region between D1S198 and D1S219 in the GB4 Whitehead radiation hybrid map published in GeneMap ’99 in the NCBI database; a length of approximately 390 kb. As described previously [8], genomic DNAs were amplified by PCR in the presence of [α-32P]dCTP, electrophoresed in 7% acrylamide denaturing gels with 10% or 0% glycerol, and exposed to X-ray film. All members of a family were run side by side in the same gel. DNAs of the probands of families that could not be ruled out from segregating with a particular gene were screened by SSCP/E as described previously [8]. Primers used to amplify the exons were based on flanking intronic sequences published in GenBank (PDE6B: X62691-X62695; PDE6A: AH006685 and U39147-U39188; RPE65: AF039856-AF039868 and U20476, U20477-U20482, U20484-U20486, U20510). The PDE6A sequences were kindly provided by Dr. Steven Pittler (University of Alabama at Birmingham) before they were published, and the RPE65 primers used were those described by Gu et al. [13]. A list of primer sequences is available upon request. Any variant exons were prepared for sequencing as follows. DNA was extracted from a low melt agarose gel with the QIAEXII kit following the manufacturer’s procedure (Qiagen, Chatsworth, CA). Exons were sequenced directly by thermal cycle sequencing following the Thermo Sequenase™ protocol (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) on an ABI sequencer (Applied Biotech, Foster City, CA).

Clinical evaluations of the patients from families with an RPE65 deletion were performed at two sites: in Sardinia and in Philadelphia. At the first site, fundus images were documented in color conventionally and with a Heidelberg Retina Angiograph (Heidelberg Engineering GmbH, Heidelberg, Germany) for green and infrared images. Electroretinograms (ERGs) and dark adaptometry were performed as previously described [26]. At the second site, standard and small-signal ERGs were performed according to published protocols [27]. Also at the second site, dark- and light-adapted static threshold perimetry and bleaching adaptation were performed with a modified automated perimeter [28]. For bleaching adaptation, 500 and 650 nm stimuli (1.7° in diameter, 200 ms in duration) were tested at a locus 30° in the temporal field [29]. Subjects had pupils dilated, were fully dark-adapted and then had pre-bleach thresholds determined. Next, white flashes estimated to bleach either 12% or 97% of rhodopsin were presented and thresholds determined until prebleach values were attained. Informed consent for all procedures was obtained from subjects after the nature of the studies had been explained fully.

**RESULTS**

*Mutation studies:* With the use of haplotype analysis, retinal degeneration was ruled out from segregating with the PDE6A gene in 6/14 families, with PDE6B in 11/14 families and with RPE65 in 4/13 families (the 14th family was not screened). Exon screening revealed only a few sequence variants. None were detected in the three probands screened for the 22 exons of PDE6B, only one variant in the eight probands screened for the 22 exons of PDE6A and three variants among the nine probands screened for the RPE65 gene exons. The PDE6A and two of the three RPE65 variants were common polymorphisms. However, the third RPE65 variant was significant: a 20 bp deletion in exon 4. Sequence analysis showed that the missing nucleotides corresponded to bp 344 to 363 of the RPE65 cDNA (GenBank Accession number U18991). The deletion creates a frame-shift starting with codon 97 and produces a nonsense mutation 23 codons downstream. This pro-

---

**Figure 1.** Inheritance of the 20 bp RPE65 deletion. A. Pedigree of a Sardinian family with the 20 bp RPE65 deletion. Filled symbols represent affected patients; unfilled symbols represent unaffected family members. B. Agarose gel (2%) of PCR-amplified products of RPE65 exon 4 stained with ethidium bromide; reverse image. Affected siblings are all homozygous for the lower molecular weight band while the one unaffected sibling and the father are heterozygous; DNA was not obtained from the mother. The three bands in the heterozygotes represent the normal allele, the deleted allele, and a heterodimer of the two alleles which often forms during the late stages of PCR.
duces a truncated protein of 120 amino acids with the last 23 being unrelated to the original RPE65 sequence. Therefore, the predicted protein product of this mutant gene, if it were stable, would lack 413 amino acids (77% of its original length) and have a tail of 23 different amino acids, 19% of its truncated length (23/120). Agarose gel electrophoresis of PCR amplicons of exon 4 (Figure 1) showed that the three affected siblings of the single family carrying this deletion were homozygous for the deleted allele. The fourth sibling and the father were heterozygous. A sample was not obtained from the mother. In addition, this deletion of exon 4 was shown to be absent from 60 North American and 25 Sardinian controls (170 chromosomes) by SSCPE. All controls were unrelated adults with no reported retinal disease. This ruled out the presence of this disease because it starts in childhood. Affecteds from the 14th family, and two other Sardinian arRP families were also negative for the deletion.

Clinical evaluation of patients: The original subjects included in this study were affected members of 14 families with definite or presumed autosomal recessive forms of retinal degeneration. Two other autosomal recessive retinal degeneration families (mentioned above) were also evaluated. Of the 16 families, 15 were identified as having RP based on diagnostic criteria described previously [30,31]. The 16th family (carrying the RPE65 deletion) has a sibship of three affected and one unaffected siblings (Figure 1). They come from a village in the Northwest of Sardinia. The parents of the affected family were second cousins and had no visual symptoms throughout their lives. The father died at 92 years of age, and the mother is still alive. By history, all three affected siblings were considered to have a form of retinal degeneration in early childhood and were referred to an ophthalmologist because of nystagmus and poor visual acuity. Their fundus appearance was noted to be “abnormal,” and progression to severe central and peripheral visual loss was reported in their teenage years. Their diagnosis is a severe retinal dystrophy with onset in infancy or early childhood.

On examination (at age 60), patient III-2 had no light perception, showed nystagmus and strabismus, and cortical and posterior subcapsular cataract. There was a wide area of chorioretinal atrophy including the posterior pole and the peripapillary area. Pigmentary retinopathy was evident at the posterior pole and in the periphery. Retinal vessels were attenuated, and the optic disc appeared pale (Figure 2A). Retinoscopy showed myopia and astigmatism (-8 -1 x 180°), and the ERG showed no detectable responses to conventional stimuli.

Patient III-3 (age 50 at examination) had some light perception associated with nystagmus and exotropia. Slit lamp examination revealed cortical and posterior subcapsular cataract, and the fundus appearance was similar to that of patient III-2 (Figure 2B,C). Retinoscopy showed myopia (-7 sph), and there was no detectable ERG. Patient III-4 (not examined) is reported to have only light perception. Both patients III-2 and III-3 stated that they stayed at home for most of their lives and attended a school for the blind where they learned Braille. They reported that their visual field narrowed with time, and that less light was perceived.
Three molecularly proven heterozygotes in the family (III-1 and two others not displayed on pedigree) were examined with dark adaptometry; the results were within normal limits. A standard ERG in III-1 was also normal.

Comparison of phenotype with another family with the 20 bp deletion in RPE65: The 20 bp deletion in RPE65 has also been reported recently in both alleles of a South American patient diagnosed with LCA [16,22]. The pedigree (Figure 3A) indicates parental consanguinity. There is no evident connection between this family and the one from Sardinia (family names in the South American genealogy do not appear in the Sardinian genealogy and vice versa). The ancestors of the South American family are from Spain. Nevertheless, a more ancient founder for this mutation is still possible.

Patient IV-1 has been followed by clinical examinations from early childhood until the present time (now age 12 years). Nystagmus and reduced vision were present in infancy and the child has always shown a preference for well-lit environments. Visual acuities have been 20/200 on various visits; retinoscopy has been +0.75 in both eyes. Results at age 11 indicated reduced kinetic visual field extent, markedly elevated dark- and light-adapted thresholds, and a severely abnormal but detectable cone flicker ERG [22]. Slit lamp examination has revealed no cataracts; ophthalmoscopy has mainly shown attenuated retinal vessels but no pigmentary retinopathy. At her most recent examination at age 12, subtle pigmentary abnormalities were noted both centrally and in the near periphery (Figure 3B).

The heterozygous parents (III-1 and III-2; current ages of 39 and 37 years, respectively) had no abnormalities on clinical examination. As we previously reported, their kinetic visual fields, dark- and light-adapted perimetry, and standard

![Figure 3. South American family. A. Pedigree of a South American family with the same 20 bp RPE65 deletion. Filled symbol represents affected patient; unfilled symbols, unaffected family members. B. Fundus photograph of the homozygote (patient IV-1) shows slight attenuation of the retinal vessels and mild retinal pigment epithelial changes centrally and in the periphery.](image)

![Figure 4. Visual function tests in the South American family. Visual function in the heterozygous parents and homozygous affected daughter from pedigree of Figure 3. A. Rod, mixed cone-rod, and cone electroretinograms in the heterozygous parents (left and middle columns) and in the homozygote (right column). A special protocol was used to record small signals in response to flicker (29 Hz) stimulation in the patient. Vertical bars indicate stimulus onset; calibrations are below and to the right of responses. B. Dark adaptometry results at 30 degrees temporal field in the heterozygous parents after partial (12% rhodopsin) and full (97% rhodopsin, 99% of L/M cone pigment) bleach tests with a 500 nm stimulus. The first 8 min following the full bleach (inset) was tested with the 650 nm stimulus to analyze the L/M-cone recovery. The pre-bleached baseline thresholds are marked with “PB”. Connected symbols are the data from heterozygotes; gray lines delimit range of normal results.](image)
ERGs were within normal limits [22]. In Figure 4A, the normal ERG waveforms for heterozygotes III-1 and III-2 are displayed adjacent to the very abnormal responses from their daughter (IV-1) at age 12. To explore further the functional consequences of the heterozygous state of this RPE65 mutation, we studied dark adaptation kinetics. Kinetics of rod sensitivity recovery (dark adaptation) following partial or full bleaches were normal or near-normal in both heterozygotes (Figure 4B) while kinetics of long- and middle-wavelength sensitive (L/M) cone recovery tested following the full bleach were also normal (Figure 4B, inset).

**DISCUSSION**

The 20 bp deletion in exon 4 of the RPE65 gene in the Sardinian and South American families is predicted to produce a reading frame shift and the synthesis of a truncated polypeptide that would lack 77% of the normal RPE65 protein including 23 different amino acids at the C-terminal. Therefore, it is extremely unlikely that this truncated polypeptide would be functional if it was synthesized at all (null mutations frequently result in degraded message [32]). In addition, the presence of this deletion in both alleles co-segregates with severe and early-onset retinal disease in these small families. Several literature reports cite homozygous or compound heterozygous RPE65 mutations associated with cases of severe, autosomal recessive retinal degeneration [12-16]. Therefore, it is very likely that this homozygous deletion causes retinal disease in these families.

The comparison of the two families with the 20 bp deletion in RPE65 is of interest since there are major age differences in homozygotes and this provides a glimpse of the natural history of the disease. All homozygotes had severe visual impairment early in life and when there was ERG evidence, both rod and cone functions were markedly abnormal. History, vision became progressively worse with measurable but very reduced visual acuities and limitation of visual fields in the first decade of life [22] progressing to no light perception by the sixth and seventh decade. Ophthalmoscopic findings were minimal in childhood but after decades of disease, severe pigmentary abnormalities and atrophy were present throughout the retina. The clinical diagnostic category of the patients would be either LCA or CSRD. As greater numbers of patients with RPE65 mutations are identified, complete descriptions of their phenotype and natural history should lead to better understanding of the relation between disease expression and genotype. Of specific importance to this subset of early-onset retinal degenerations will be the determination of whether or not there is an early disease stage when retinal structure is retained despite severely impaired retinal function [22], such as occurs in animal models with mutations in RPE65 [21].

Our studies of heterozygotes from both families showed no measurable abnormalities by standard ERGs and psycho-physical testing. Of interest, these results differ from those in a 50 year old heterozygote with the 65+5G→A RPE65 mutation who was reported to have dark-adapted perimetric defects, ERG abnormalities and fundus lesions [33]. Clinical and ERG examinations of other RPE65 heterozygotes have been reported to be normal [34]. In heterozygotes for the 20 bp deletion mutation in RPE65, the normal kinetics of rod sensitivity recovery following both partial and full bleaches suggests that visual pigment regeneration reactions are not rate-limited by the amount of RPE65 in man. The slow rate of 11-cis-retinal formation observed in Rpe65+/− mice [22] may point to interspecies differences in the retinoid cycle. Further support for this conjecture comes from the recent demonstration of slow cone deactivation kinetics in Grk1−/− mice [35], which is much exaggerated compared to the same physiological abnormality previously described in human patients lacking rhodopsin kinase [36]. Retinoid cycle differences may also explain interspecies variation in visual pigment regeneration kinetics [37-39].

The only one out of 16 autosomal recessive retinal degeneration families distributed throughout Sardinia (these include the three families screened only for the deletion in exon 4) that shows a disease-causing RPE65 mutation was found to be the only family with a diagnosis of CSRD or LCA. Affected individuals from the other 15 families had later-onset disease and a diagnosis of arRP. This family originates from an area of Sardinia distinct from all of the other families, suggesting that it is of an origin distinct from the other 15. In addition, the majority of cases of autosomal recessive, non-syndromic, retinal degeneration in Sardinia are classified as arRP while CSRD and/or LCA are generally uncommon [30].

**ACKNOWLEDGEMENTS**

This work was supported by grants from the National Institutes of Health (EY08285, EY05627), the Foundation Fighting Blindness, the Stephen Wynn and Elaine Wynn Charitable Foundation, and the British Retinitis Pigmentosa Society. DBF is a recipient of a Research to Prevent Blindness Senior Scientist Investigators award. We are grateful to Daniel A. Marks for help with data and figures, and David B. Hanna, Elaine de Castro and Jessica Emmons for clinical coordination.

**REFERENCES**


