RPE65 gene: multiplex PCR and mutation screening in patients from India with retinal degenerative diseases

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Abstract

We used multiplex PCR followed by sequencing to screen for mutations in the 14 exons of the *RPE65* gene in early-childhood-onset autosomal recessive retinitis pigmentosa (arRP) and Leber's congenital amaurosis (LCA) patients. The RPE65 protein is believed to play an important role in the metabolism of vitamin A in the visual cycle and mutations identified in the gene could have implications for vitamin A-based therapeutic intervention. We were able to identify a homozygous mutation (AAT → AAG) in exon 9 in an arRP patient and a heterozygous missense transversion (AAT → AAG) also in exon 9 of an LCA patient. We also identified a polymorphism in exon 10 (GAG → GAA) in an arRP as well as an LCA patient. Mutation screening would be greatly facilitated by multiplex PCR which could cut down costs, labour and time involved. The nucleotide changes observed in this study could be *de novo*. Though a larger study has been undertaken, from the preliminary results it appears that in India the *RPE65* gene seems to be less involved in causation of LCA.

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Introduction

Polymerase chain reaction (PCR) coamplification of several exons/introns of one or more genes in a single reaction vial is called multiplexing (Chamberlain *et al*. 1990; Mullis *et al*. 1994). A time-saving, labour-saving and cost-effective technique, multiplexing could be of considerable use in studies such as mutational screening of genes where several exons are required to be amplified and sequenced. There has been extensive application of this technique for mutational screening in earlier studies: Bor *et al*. (2001) used multiplexing to screen for microdeletions in the human Y chromosome; Effat *et al*. (2000) applied a rapid multiplex PCR method to screen for mutations in the dystrophin gene in patients of Duchenne's muscular dystrophy/Becker's muscular dystrophy; Dreesen *et al*. (2000) employed a multiplex PCR of polymorphic markers flanking the *CFTR* gene for preimplantation genetic diagnosis of cystic fibrosis; Scheffer *et al*. (2000) have applied multiplex PCR for identifying mutations in the retinoblastoma gene.

We have used multiplexing to screen for mutations in the *RPE65* gene in Indian patients of autosomal recessive retinitis pigmentosa (arRP) and Leber's congenital amaurosis (LCA). The *RPE65* gene has 14 exons and codes for an evolutionarily conserved 61-kDa protein associated with smooth endoplasmic reticulum of the retinal pigment epithelium (RPE) (Nicoletti *et al*. 1995). The RPE65 protein is believed to play an important role in the metabolism of vitamin A and the generation of the chromophore 11-*cis*-retinal in the visual cycle (Redmond *et al*. 1998). Mutations in *RPE65* appear to account for approximately 2% of cases of early-childhood-onset arRP and 16% of cases of LCA (Morimura *et al*. 1998). Characterization of the mutation spectrum of the *RPE65*

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gene in these patients assumes considerable relevance in the context of a recent report wherein Swedish briard/briard-beagle dogs with retinal dystrophy due to a deficiency in RPE65 activity owing to a *RPE65* –/– genotype (Veske *et al*. 1999) were successfully treated by gene therapy using adeno-associated viral vectors (Acland *et al*. 2001).

With the completion of sequencing in the human genome project and mapping of many genes, DNA diagnostics has received a new fillip. There will be more genes sequenced for diagnostics, which might be done prenatally or postnatally or presymptomatically or postsymptomatically. Whatever the reason, accurate but rapid diagnostic procedures would be widely applied in medical, paediatric and genetic clinics all over the world to assist in the management of patients with Mendelian and complex diseases like cystic fibrosis, retinitis pigmentosa, and breast and colon cancers.

In this paper we report *RPE65* mutations in a small sample of arRP and LCA patients. This gene has potential therapeutic application in the near future. We also note the advantage of multiplex PCR in mutation screening.

Materials and methods

In the present study mutation screening of the *RPE65* gene was conducted on one arRP and nine LCA patients using multiplex PCR followed by DNA sequencing. Venous blood samples and family information for the present investigation were obtained with informed consent of the patients.

Ophthalmic diagnostic tests undertaken to confirm RP or LCA included Humphrey's perimetry colour vision tests, fundus examination and electroretinogram (ERG). Fundus examination and electroretinogram were done under general anaesthesia. Patients with arRP present with a marked history of diminished vision. Characteristic fundus features observed include normal or pale optic disc, arteriolar attenuation, pigmentary alterations, mottled appearance of retinal midperiphery and an extinguished ERG. LCA patients report marked history of loss of vision since birth, nystagmus and also history of oculodigital reflex (squeezing eyes). They have hyperopic refractive error and the fundus appearance may vary from normal to RP-like features. ERG response is an extinguished waveform.

The determination of an autosomal recessive inheritance of the disease trait was ascertained from detailed pedigree based on information from the proband and accompanying relatives. The criteria for ascertaining autosomal recessive pattern were (a) both males and females affected and (b) parents unaffected.

Laboratory methods involved isolation of DNA from venous blood samples using the standard phenol/chloroform extraction method (Wolff and Gemmil 1997) followed by multiplex PCR for all 14 exons of the *RPE65* gene. Primer sequences were obtained from earlier published results (Gu *et al*. 1997). The PCR products were electrophoresed in 2% agarose gel with ethidium bromide stain and visualized under UV light in a transilluminator (Amersham Pharmacia). The amplified products that appear as bright fluorescing bands were then cut from the gel and DNA eluted using QIAEX II gel extraction kit (Qiagen). The eluted DNA was then used for cycle sequencing PCR using fluorescent dNTPs (Ready Reaction Mix) and then sequenced in the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Identification of mutations was done by comparing sample sequence with a reference sequence from GenBank (http://www.ncbi.nlm.nih.gov).

In our laboratory we had previously standardized PCR conditions for all exons of the *RPE65* gene as individual reactions. For multiplexing we assembled these exons into five groups depending on the commonality of annealing temperatures and sufficiently different product size. It is essential that amplicon sizes be different to allow for adequate separation during electrophoresis. Thereafter parameters like primer concentration, annealing temperature and time, number of cycles, etc. were varied till amplified products could be obtained with good yield. For all amplifications a 20-µl reaction mixture was prepared using 100 ng of genomic DNA, 0.2 µl of each primer (20 pM), 4 µl dNTPs (40 nM), 4 µl PCR buffer and 1.2 µl *Taq* DNA polymerase. The PCR was carried out in the PE 2400 thermal cycler. Exons 11, 12 and 13 had almost similar annealing temperatures, that is 48°C, 47°C and 46°C respectively, but their product sizes were very close making ideal separation in 2% agarose impossible. So the PCR for these exons was standardized at annealing temperature of 47°C as separate reactions in a single PCR machine run. Thirtyseven normal unrelated individuals were also sequenced and analysed as controls.

Results

Annealing temperature, product size and standardized PCR conditions for all five groups of exons for multiplexing are given in table 1. Figure 1 shows the electrophoresed multiplex PCR products.

In one patient of arRP a homozygous mutation in exon 9 was identified. This transversion (AAT → AAG) occurred at nucleotide 1017 in codon 321 of the gene, which results in change from asparagine to lysine (N321K) (figure 2). The pedigree of this patient with autosomal recessive inheritance is shown in figure 3. In an LCA patient a heterozygous missense transversion (AAT → AAG) in exon 9 was identified in the same codon 321, which also results in change from asparigine to lysine. A hetero-

zygous polymorphism (GAG → GAA) at the nucleotide 1110 in exon 10 was observed in an LCA patient as well as an arRP patient; this does not result in an amino acid change. None of these changes was observed in the 37 control samples.

Discussion

RPE65 expression is seen specifically in retinal pigment epithelium and not in any other tissues like brain, heart, liver, lung and small intestine by Northern blot assays (Hamel *et al*. 1993). RPE65 protein has a vital role in the vitamin A cycle in the retina. Overaccumulation of *all-trans* retinyl esters, lack of 11-*cis*-retinol, association of RPE65 protein with retinol dehydrogenase and degeneration of rhodopsin in these conditions in presence of normal opsin suggest active role of RPE65 in the visual cycle relating to maintenance of rhodopsin. Gu *et al*.

Table 1. Multiplex groups of *RPE65* exons and PCR conditions.

			PCR conditions						
			Denaturation** temp., 94°C time, 45 sec	Annealing			Extension		
Multiplex group	Exons	Product size (bp)	Cycles	Temp.	Time	Cycles	Temp.	Time	Cycles
I	1 7 and 8	244 545	35	54°C	60 sec	35	72°C	90 sec	35
II	2	282		Cycle I					
				53°C				30 sec	
	3	199	8	52°C	30 sec	8	72°C	1 min	8
				48°C				1 min	
	14	453		Cycle II					
				53°C				30 sec	
			5	52°C	30 sec	5	72°C	1 min	5
				48°C				1 min	
III	4 and 5 6	501 269	35	54°C	60 sec	35	72°C	90 sec	35
IV	9 10	292 226	35	55°C	60 sec	35	72°C	90 sec	35
V*	11 12 13	197 176 199	35	47°C	60 sec	35	72°C	90 sec	35

*Set as separate reactions.
**Preceded by an initial denaturation step of one cycle at 94°C for 5 min.

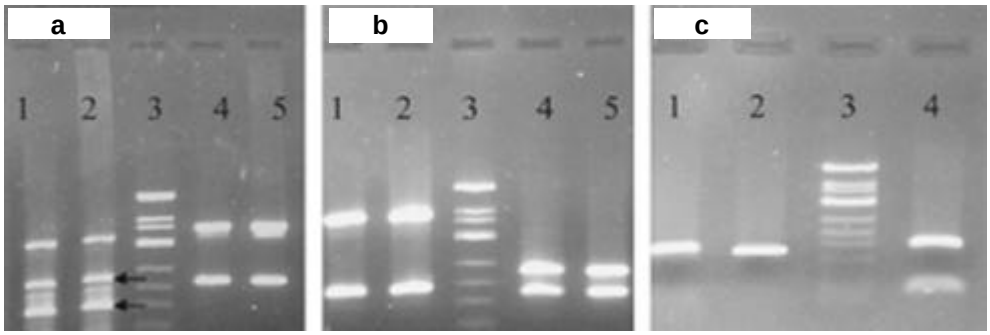

Figure 1. Multiplex PCR products of *RPE65* gene exons. (a) Lanes 1 and 2, exons 2 (199 bp), 3 (282 bp) and 14 (453 bp); lane 3, marker (*Hin*fI digest); lanes 4 and 5, exons 4 and 5 (501 bp) and 6 (262 bp). (b) Lanes 1 and 2, exons 1 (244 bp) and 7 and 8 (545 bp); lane 3, marker (*Hin*fI digest); lanes 4 and 5, exons 9 (292 bp) and 10 (226 bp). (c) Lane 1, exon 11 (197 bp); lane 2, exon 12 (176 bp); lane 3, marker (*Hin*fI digest); lane 4, exon 13 (199 bp).

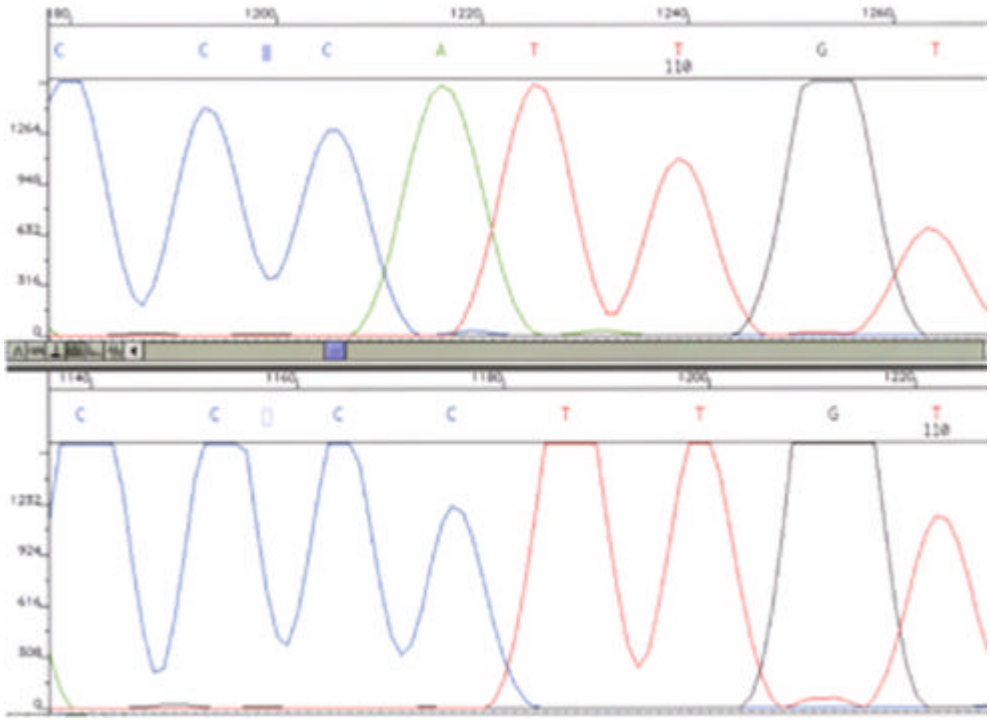

Figure 2. Homozygous mutation in *RPE65* exon 9, detected by automated fluorescent sequencing method with ABI Prism 310 DNA sequencer. Top, *RPE65* exon 9 wild-type sequence using reverse primer; bottom, exon 9 sequence in arRP patient using reverse primer, indicating A → C transversion.

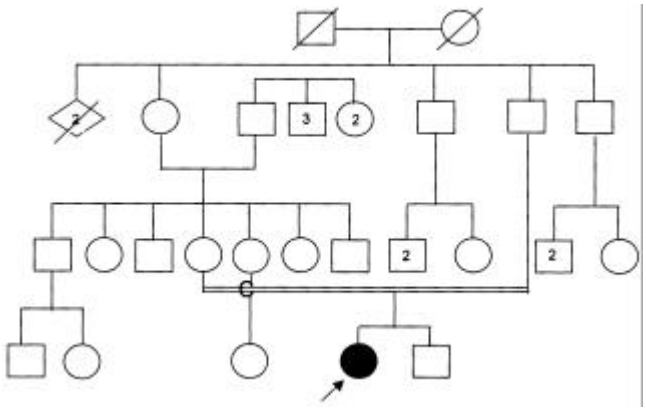

Figure 3. Pedigree of an arRP patient with homozygous AAT → AAG mutation.

(1997) found many splice-site mutations, missense mutations and other mutations that affect protein function. Marlhens *et al*. (1997) studied 12 patients with LCA and reported mutations in *RPE65*. Morimura *et al*. (1998) studied 147 unrelated patients with arRP, 15 with isolated RP and 45 with LCA. Mutations considered to cause pathology were found in these patients in *RPE65*, accounting for ≈ 2% of cases of recessive RP and ≈ 16% of cases of LCA.

Lorenz *et al*. (2000) screened four children from three families with severe visual handicap from infancy and found mutations in *RPE65* in homozygous state. The parents of the affected children when screened revealed heterozygous status for the mutation. Mutations in *RPE65* have been shown to be the cause of arRP and LCA. Missense mutations, point mutations affecting splicing, insertions and deletions have been reported.

In our study one arRP patient showed a homozygous mutation and one LCA patient showed a heterozygous mutation in exon 9, which causes change from asparagine to lysine (AAT → AAG), at nucleotide 1017 (codon 321). The T → G change is a transversion that substitutes the acidic, polar amino acid asparagine with the basic lysine. This mutation could result in altered protein tertiary structure, affecting its function. Of the one arRP and nine LCA patients only the former had a pathological mutation and it could be *de novo*. This is a preliminary result of a larger ongoing screening project, and presently we find that in India *RPE65* may not have a major role in causation of LCA.

The recognition of specific mutations in a gene could unravel the underlying molecular basis of the disease, which could facilitate counselling and possible future therapeutic interventions. In this regard use of multiplex PCR, especially in coordination with additional techniques like secondary structural content prediction (SSCP) and

density gradient gel electropheresis (DGGE), could increase the rapidity with which diagnosis could be made.

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