Mutation analysis of Leber congenital amaurosis-associated genes in patients with retinitis pigmentosa

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Abstract. The genetic defects underlying approximately half of all retinitis pigmentosa (RP) cases are unknown. A number of genes responsible for Leber congenital amaurosis (LCA) may also cause RP when they are mutated. Our previous study revealed that variants in the most frequently mutated nine exons accounted for approximately half of the mutations detected in a cohort of patients with LCA. The aim of the present study was to detect mutations in LCA-associated genes in patients with RP using two different strategies. Sanger sequencing was used to screen mutations in the nine exons in 293 patients with RP and exome sequencing was used to detect variants in 12 LCA-associated genes in 157 of the 293 patients with RP and then to validate the variants by Sanger sequencing. Potential pathogenic mutations were identified in four patients with early onset RP, including homozygous *CRB1* mutations in two patients, compound heterozygous *CRB1* mutations in one patient and compound heterozygous *CEP290* mutations in one patient. The present study indicated that mutations in *CEP290* may also be associated with RP but not with LCA. With the exception of *CEP290*, the remaining 11 genes known to be associated with LCA but not with RP are unlikely to be a common cause of RP.

Introduction

Retinitis pigmentosa (RP) is the most common form of progressive hereditary retinal degeneration, with a worldwide prevalence of ~1 in 4,000 (1,2). To date, mutations in at least 61 genes have been reported to cause RP (RetNet, https://sph.uth.edu/Retnet/). However, mutations in these genes contribute to only half of the clinical cases (3). Therefore, identification of additional genes responsible for RP is important to determine the molecular basis of RP and aid in the development of novel therapeutic strategies. Genes known to cause other forms of hereditary retinal degeneration may be good candidates for genes causing RP.

Leber congenital amaurosis (LCA) is the most severe form of hereditary retinal degeneration, with ~20 causative genes identified. Our previous study has shown that about half of the variants were detected in nine frequently mutated exons (4). Mutations in eight of the 20 LCA-associated genes have been reported to cause RP as well (4-7). However, systemic evaluation of LCA-associated genes in patients with RP is limited (8-10), particularly for those 12 of the 20 genes in which a mutation has not been identified in patients with RP. The 12 genes known to cause LCA but not RP are as follows: Aryl hydrocarbon interacting protein-like 1 (*AIPL1*) (11), calcium-binding protein 4 (*CABP4*) (12), centrosomal protein 290 kDa (*CEP290*) (13), death domain containing 1 (*DTHD1*) (14), guanylate cyclase 2D, membrane (retina-specific; *GUCY2D*) (15), IQ motif-containing protein B1 (*IQCB1*) (16), potassium inwardly-rectifying channel, subfamily J, member 13 (*KCNJ13*) (17), Leber congenital amaurosis 5 (*LCA5*) (18), nicotinamide nucleotide adenylyltransferase 1 (*NMNAT1*) (19), orthodenticle homeobox 2 (*OTX2*) (20), retinal degeneration 3 (*RD3*) (21) and retinitis pigmentosa GTPase regulator interacting protein 1 (*RPGRIP1*) (22).

In the present study, variations in LCA-associated genes were evaluated in a cohort of patients with RP using two methods: i) The most commonly mutated nine exons were analyzed by Sanger sequencing in 293 patients with RP; and ii) for the 12 genes known to associate with LCA but not RP, variants that resulted from exome sequencing in 157 of the 293 patients with RP were selected and then further confirmed by Sanger sequencing. Mutations in four patients with RP were identified in LCA-associated genes.

Subjects and methods

Subjects. Probands with a clinical diagnosis of RP from 293 unrelated families were recruited from the Pediatric and Genetic Eye Clinic, Zhongshan Ophthalmic Center (Guangzhou, China) since 1996. The diagnosis of RP was based on phenotypes described in a previous study (23). Written informed consent from each participant or their guardians was obtained prior to collection of their clinical data and venous blood samples.

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Table I. Most frequently mutated nine exons analyzed.

Gene	OMIM	Map location	Number of coding exons	Targeted exon(s)	Frequency for mutant alleles in Chinese LCA patients (4), %
GUCY2D	600179	17p13.1	18	2	9.00
				11	3.85
				12	3.85
CRB1	604210	1q31-q32.1	12	6	7.70
				9	5.14
				11	6.41
RPE65	180069	1p31	14	4	5.14
RPGRIP1	605446	14q11	24	3	5.14
CEP290	610142	12q21.32	53	6	3.85

OMIM, Online Mendelian Inheritance in Man; LCA, Leber congenital amaurosis.

Genomic DNA was prepared from leukocytes in venous blood as previously described (24). The present study was approved by the Institutional Review Board of the Zhongshan Ophthalmic Center.

Analysis of the nine frequently mutated exons. The most frequently mutated nine exons were selected based on our previous study (4), as listed in Table I. The genomic fragments of the nine exons were amplified by polymerase chain reaction, using primers encompassing each of the nine exons and the adjacent intronic regions (Table II). Touchdown PCR amplifications of the genomic fragments, sequencing and result analysis was processed as previously described (4).

Variants in 12 genes as determined by exome sequencing. Whole exome sequencing was performed on 157 of the 293 unrelated patients with RP, using a commercial service from BGI Shenzhen (Shenzhen, China; http://www.genomics.cn/index) as previously described (25,26). Mutations in 60 genes responsible for RP were identified in approximately half of these patients (27). The variants in the 12 genes known to be associated with LCA but not RP, resulted from exome sequencing of the 157 patients with RP, were collected for further analysis. Heterozygous variants for dominant genes and homozygous or compound heterozygous variants for recessive genes were selected and verified by Sanger sequencing, using primers to amplify the individual fragments harboring variants (Table III). The mutation hot spot, c.2991+1655A>G in *CEP290* (13), is at the position beyond the scope of exome sequencing, thus genomic fragments of *CEP290* encompassing c.2991+1655A>G were amplified and analyzed by Sanger sequencing in all 157 RP patients.

Bioinformatics analysis. In total, two online computational prediction algorithms, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/), were used to predict the functional impact of missense mutations identified (28). The PolyPhen-2 website can predict the functional effect of variants and classify them into 'probably damaging', 'possibly damaging', 'benign', and 'unknown' (29). The SIFT algorithm shows a normalized probability score of a missense variant: When the normalized probability is larger than 0.05, the variant is predicted to be 'tolerated', otherwise, the variant is predicted to be 'damaging' (30). The impact of variants on the splice site was predicted by NNSPLICE version 0.9 (http://fruitfly.org/seq_tools/splice.html) (7). The description of variants referred to the nomenclature of the Human Genomic Variation Society (http://www.hgvs.org/mutnomen/). Novel variants were further evaluated in 192 normal controls.

Results

Sanger sequencing. Analysis of the nine frequently mutated exons identified potential pathogenic mutations in the *CRB1* gene in three patients, including one known and two novel mutations, i.e. c.1831T>C (p.S611P), c.1841G>T (p.G614V) and c.3442T>C (p.C1148R) (Table IV; Fig. 1). These variants were not present in the 192 unaffected controls. One patient had compound heterozygous mutations and the other two had homozygous mutations (Table V).

Exome sequencing. Exome sequencing identified six variants in two of the 12 genes, including four variants in *CEP290* and two variants in *LCA5*, i.e. c.[442-10_11insT];[6736A>G] in *CEP290* of RP397, c.[4040G>A];[3104-2delA] in *CEP290* of RP276, and c.[1642C>T];[634G>T] in *LCA5* of RP374. The compound heterozygous c.[4040G>A];[3104-2delA] mutations in *CEP290* of RP276 were considered to be potential pathogenic mutations (Tables IV and V, Fig. 1). The other two compound heterozygous variants in *CEP290* and *LCA5*, respectively, were unlikely pathogenic since the c.6736A>G (p.K2246E) in *CEP290* and the c.1642C>T (p.P548S) in *LCA5* were predicted to be benign or tolerated by PolyPhen-2 and SIFT, while mutations in these two genes are associated with recessive retinal diseases. No potentially pathogenic variants were detected in the remaining 10 of the 12 genes, including *AIPL1*, *CABP4*, *DTHD1*, *GUCY2D*, *IQCB1*, *KCNJ13*, *NMNAT1*, *OTX2*, *RD3* and *RPGRIP1*.

Clinical data of patients with LCA-associated gene mutations. Clinical data of the four RP patients with mutations in the LCA-associated genes were summarized in Table V. The patients examined were between 5 and 29 years old. Although they presented with poor vision or night blindness,

Table II. Primers used for the most frequently mutated nine exons.

Primer name	Primer sequence, 5'-3'	Size of amplicon, bp
GUCY2D-E2a-FW	ccttggccccagttagtctt	552
GUCY2D-E2a-RV	gttcaccggacccacgag	
GUCY2D-E2b-FW	gtccccgcttcgaggtag	552
GUCY2D-E2b-RV	accgagtgcatcaccatga	
GUCY2D-E11-FW	gatagttgcagggctggtct	397
GUCY2D-E11-RV	gtttcatcactgggctttgc	
GUCY2D-E12-FW	tgaacctctgatgtaaagaaacc	398
GUCY2D-E12-RV	gtagcctggaaggccagag	
CRB1-E6a-FW	ttcatgcacttctgcaagatt	598
CRB1-E6a-RV	tgaacagaagcacctttgactg	
CRB1-E6b-FW	cgaagcaacagggatgtgtt	648
CRB1-E6b-RV	tttcatagcaggcagaagca	
CRB1-E9a-FW	atgtatcaaatagtcaatatgcaatgt	598
CRB1-E9a-RV	gagataaatgcctccgatttc	
CRB1-E9b-FW	tgtgggagacagagctattga	594
CRB1-E9b-RV	cttgaggagagagctttccaa	
CRB1-E11-FW	agactgtgctgttccagagaga	344
CRB1-E11-RV	ctgttcaccccactcaacaa	
RPE65-E4-FW	ccctttattcttcatgttgtgc	380
RPE65-E4-RV	gtcagtaacctctactcctcgaaa	
RPGRIP1-E3-FW	tgtggttaatagatcacggtagatg	530
RPGRIP1-E3-RV	gcagaaaggagggagtgaga	
CEP290-E6-FW	gcttgttgttgactcatttgaa	375
CEP290-E6-RV	ttggtgatgacaaaatgaaca	

FW, forward; RV, reverse; bp, base pairs. Annealing temperature, 58-65˚C.

Table III. Primers used for validating variants from exome sequencing.

Primer name	Primer sequence, 5'-3'	Target variant/sequenced sample
CEP290-E7-FW	tttgaaaattttggcctattatttatg	*CEP290:c.442-10_11insT*/RP397
CEP290-E7-RV	tccctgagacaaagtcatacca	
CEP290-IVS26+1655-FW	ggttcaggccgttctcct	*CEP290:c.2991+1655A>G*/All patients
CEP290-IVS26+1655-RV	agtttttaaggcggggagtc	
CEP290-E28-FW	tccaggtctgatggaattcag	*CEP290:c.3104-2delA*/RP276
CEP290-E28-RV	ttcagagatccagacaaaccac	
CEP290-E32-FW	tttgtcatgtagtttgacaaaagat	*CEP290:c.4040G>A*/RP276
CEP290-E32-RV	cggatcatgaggtcaggaga	
CEP290-E49-FW	agcatttagagccccaggtt	*CEP290:c.6736A>G*/RP397
CEP290-E49-RV	ctgttcatcaggaagaaacca	
LCA5-E4-FW	caagagaaagaacgggcaac	*LCA5:c.634G>T*/RP374
LCA5-E4-RV	atgcccaatgagaaacatcc	
LCA5-E9-FW	ccagagagaagccccaaaac	*LCA5:c.1642C>T*/RP374
LCA5-E9-RV	tggatttgacctctctgatgtt	

FW, forward; RV, reverse. Annealing temperature, 58-65˚C.

none of thee patients exhibited nystagmus or oculardigital sign. These patients were likely to have early onset severe RP.

Discussion

In the present study, potentially pathogenic mutations in

Table IV. Potential pathogenic mutations detected in patients with RP.

Gene	Method	Nucleotide change	Amino acid change	Bioinformatics		Status/patient ID	Allele frequency		Reported
				P/SS	SIFT		Patients	Controls	
CRB1	Sanger	c.1831T>C	p.S611P	PrD	D	Het/RP019; Hom/RP051	4/586	0/384	Li *et al* (4)
CRB1	Sanger	c.1841G>T	p.G614V	PrD	D	Het/RP019	1/586	0/384	Novel
CRB1	Sanger	c.3442T>C	p.C1148R	PrD	D	Hom/RP173	1/586	0/384	Novel
CEP290	Exome	c.3104-2delA	Splicing defect	SSA	NA	Het/RP276	1/314	0/192	Novel
CEP290	Exome	c.4040G>A	p.W1347*	NA	NA	Het/RP276	1/314	0/192	Novel

RP, retinitis pigmentosa; P/SS, Polyphen-2/Splice Site Prediction; SIFT, Scale-invariant feature transform; ID, identity; Het, heterozygous; Hom, homozygous; PrD, probably damaging; D, damaging; SSA, splicing site abolished; NA, not applicable, *stop codon.

Table V. Clinical data of the four patients with mutations.

Patient ID	Gene	Variations	Inheritance	Gender	Age (years)		First symptom	Visual acuity (OD; OS)	Fundus changes	Electroretinography response	
					Exam	Onset				Rod	Cone
RP019	*CRB1*	c.[1831T>C];[1841G>T]	AR	Male	17	EC	PV, NB	FC; FC	AV, WPD, PD, MD	NA	NA
RP051	*CRB1*	c.[1831T>C];[1831T>C]	Isolated	Female	5	3	NB, CB	0.1; 0.3	PD, MD	Severely reduced	Severely reduced
RP173	*CRB1*	c.[3442T>C];[3442T>C]	Isolated/cons	Female	29	EC	PV	HM; HM	AV, PD	NA	NA
RP276	*CEP290*	c.[4040G>A];[3104-2delA]	Isolated	Male	6	EC	NB	ND	AV, CRD	Extinguished	Extinguished

ID, identity; OD, right eye; OS, left eye; AR, autosomal recessive; EC, early childhood; PV, poor vision; NB, night blindness; FC, finger counting; AV, attenuated vessels; WPD, waxy pale disc; PD, pigment deposit; MD, macular degeneration; NA, not available;CB, color blindness; Cons, consanguinity; HM, hand movement; ND, not dectected; CRD, carpet-like retinal degeneration.

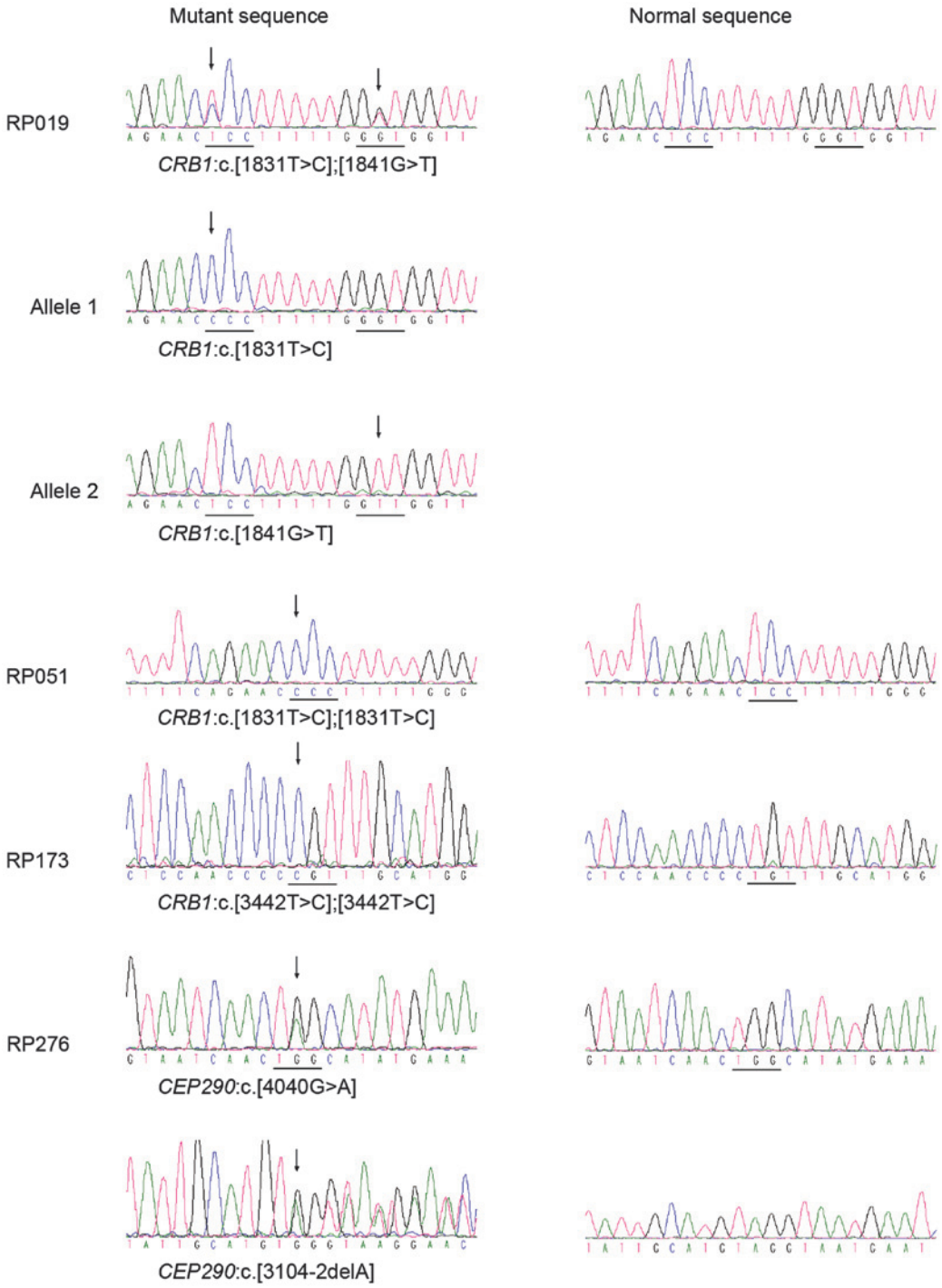

Figure 1. Sequence chromatography of the four RP probands who harbored homozygous or compound heterozygous mutations. Sequence changes detected in the RP probands (left column) are shown compared with the corresponding normal sequences (right column). The arrows indicate the mutation sites; the underlined regions indicate the codons involved in missense mutations. Compound heterozygous mutations in exon 6 of *CRB1* were detected by Sanger sequencing in the patient RP019, and these two variants were close to each other (10 bp). These compound heterozygous mutations were further analyzed by clone sequencing as described previously (30), in order to confirm that these two variants were located on different alleles. RP, retinitis pigmentosa.

LCA-associated genes were identified in four of the 293 patients with RP. By screening the most frequently mutated nine exons, homozygous or compound heterozygous mutations were identified in two exons of the *CRB1* gene in 1.0% (3/293) of RP probands. No such variant was detected in the rest of the six exons of the other four genes. This indicates that the mutation rate of these nine exons in RP patients is markedly lower compared with that in the LCA patients (4). After analyzing the variants in 12 genes, associated with LCA but not RP, resulting from exome sequencing of 157 patients, it was confirmed that one patient harbored compound heterozygous mutations in *CEP290*. Previously, the *CEP290* mutation in patients with RP has rarely been reported, except for the compound heterozygous mutations, c.[4705-1G>T];[3559delC], in an autosomal recessive RP patient (32).

All four patients with RP demonstrated early onset severe retinal degeneration, but also, they were marginally different from LCA due to the absence of nystagmus and oculodigital

signs. According to previous studies, among RP patients caused by *CRB1* mutations, patients with null mutations (i.e., nonsense, frameshift and splice-site mutations) on the two alleles are likely to result in a more severe form of retinal degeneration (e.g. LCA), while a missense mutation on at least one allele may suffer from a milder phenotype (e.g. RP) (33,34). Previous studies have revealed that *CRB1* can cause autosomal recessive RP, and it was recently reported that *CRB1* mutations are a relatively frequent cause of autosomal recessive early onset retinal degeneration in Israeli, Palestinian and Spanish populations (34,35).

Hereditary retinal degeneration is a complicated group of diseases causing blindness. For each form, including RP, LCA or cone-rod dystrophies, a number of causative genes have been identified. Sometimes, atypical phenotypes or phenotypic progression may hinder proper classification of the diseases and as a result analysis of pertinent candidate genes may not be available. Conversely, a number of genes responsible for one form of retinal degeneration may also lead to other forms of the disease. Those genes that are considered to cause a certain form of retinal degeneration may remain potential candidate genes for other forms of retinal dystrophy. Extensive analysis of all the potential candidate genes, not just a subset of well-defined known causative genes, may lead to the identification of the genetic defects in more patients with retinal degeneration. This may be increasingly significant in the era of exome sequencing.

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