Whole Exome Sequencing in Thai Patients With Retinitis Pigmentosa Reveals Novel Mutations in Six Genes

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Purpose. To identify disease-causing mutations and describe genotype–phenotype correlations in Thai patients with nonsyndromic retinitis pigmentosa (RP).

Methods. Whole exome sequencing was performed in 20 unrelated patients. Eighty-six genes associated with RP, Leber congenital amaurosis, and cone-rod dystrophy were analyzed for variant detection.

Results. Seventeen variants (13 novel and 4 known) in 13 genes were identified in 11 patients. These variants include 10 missense substitutions, 2 nonsense mutations, 3 deletions, 1 insertion, and 1 splice site change. Nine patients with identified inheritance patterns carried a total of 10 potentially pathogenic mutations located in genes *CRB1*, *C8orf37*, *EYS*, *PROM1*, *RP2*, and *USH2A*. Three of the nine patients also demonstrated additional heterozygous variants in genes *ABCA4*, *GUCY2D*, *RD3*, *ROM1*, and *TULP1*. In addition, two patients carried variants of uncertain significance in genes *FSCN2* and *NR2E3*. The RP phenotypes of our patients were consistent with previous reports.

Conclusions. This is the first report of mutations in Thai RP patients. These findings are useful for genotype–phenotype comparisons among different ethnic groups.

Keywords: retinitis pigmentosa, whole exome sequencing, genotype–phenotype correlations, mutation screening

Retinitis pigmentosa (RP) is the most commonly inherited retinal disease and is both clinically and genetically heterogeneous. The worldwide prevalence of RP is approximately 1 in 3500 to 5000.[1] The severity and age of onset of RP can vary among patients. Night blindness is usually the first symptom during adolescence with subsequent peripheral visual field loss occurring in young adulthood. In the later stages of disease, patients start to lose their central vision, usually between the ages of 40 to 60.[2] Retinitis pigmentosa presents different modes of inheritance including autosomal recessive (arRP), autosomal dominant (adRP), X-linked recessive (xlRP), and at least two rare forms, digenic and mitochondrial trait.[1]

Mutations in at least 57 associated genes distributed among all RP modes of inheritance have been identified (The Retinal Information Network [RetNet]). The proteins encoded by these genes are involved in a variety of functions, including the phototransduction cascade, vitamin A metabolism, photoreceptor structural and cytoskeletal formation, cell-to-cell signaling or synaptic interaction, RNA intron-splicing factors, intracellular protein trafficking, pH regulation, and phagocytosis.[2]

There are many factors that contribute to the complexity of RP. Different mutations in the same RP-associated gene can cause different diseases. Furthermore, clinical variability, which may be due to modifier genes, has been observed in different affected individuals both in inter- and intrafamilial cases with the same mutation.[3,4] Although many RP-associated genes have been reported, mutation screening of these genes fails to detect the disease-causing mutations in approximately 40% of RP patients.[1] Due to genetic heterogeneity and the fact that no clear genotype-phenotype correlations have been described, sequence analysis of the entire coding region for each candidate gene is the most commonly used molecular genetic test for RP. However, this method is laborious, time-consuming, and expensive. Recent developments in next-generation sequencing (NGS) represent an efficient, time-saving, and cost-effective tool for variant detection in affected individuals.[5] To date, NGS has been successfully applied to identify mutations in genes known to be associated with RP[6–16] and

has also led to the discovery of several novel candidate RP genes.[8,11–13]

In this study, we present the first mutation analysis of 20 unrelated Thai patients with nonsyndromic RP using whole exome sequencing (WES). Seventeen different variants in 13 genes were identified in 11 patients. We also describe the genotype–phenotype correlation of each patient.

Methods

Clinical Data and Sample Collection

Twenty unrelated Thai patients with nonsyndromic RP were evaluated at the Division of Ophthalmology, Siriraj Hospital, Bangkok. The ophthalmological examination included best-corrected visual acuity (BCVA) using Snellen chart, slit-lamp biomicroscopy, dilated fundus examination, optical coherence tomography (OCT, when available), full-field electroretinogram (ERG), and visual evoked potential (VEP). All patients were of Thai ancestry ranging in age from 5 to 69 years. This study was approved by the Institutional Review Board of Siriraj Hospital Mahidol University, being adherent to the Declaration of Helsinki. Peripheral blood samples were collected from all participants after informed consent was obtained. Deoxyribonucleic acid was extracted from leukocytes according to the salting-out method.[17] Ethnically matched, unrelated healthy individuals were used for normal control screening.

Whole Exome Sequencing (WES)

Exome capture was performed using solution hybrid selection with a commercial enrichment kit (SureSelect^{XT} Human All Exon Automated Target Enrichment Kit; Agilent Technologies, Santa Clara, CA, USA) for Illumina paired-end multiplexed sequencing. Exome capture libraries of 2 × 100 bp paired-end reads were sequenced on an array scanner (Illumina HiSequation 2000; Illumina, Inc., San Diego, CA, USA) at the Department of Medical Genome Sciences, The University of Tokyo. Three DNA samples were pooled on each lane of the flow cell for sequencing.

Sequence Data Alignment, Variant Calling and Identification

The Illumina paired-end DNA sequence data were mapped and aligned to the reference human genome NCBI Build 37 (hg19), using the Burrows-Wheeler Aligner (BWA) program. A set of utilities (Sequence Alignment/Map [SAMtools], a tab-delimited text file that contains sequence alignment data; Wellcome Trust Sanger Institute, Hinxton, UK) was used to convert the alignments from SAM to BAM files (the binary version of a SAM file), sort and merge the alignments, remove PCR duplicates, and index the alignment data. A software package (The Genome Analysis Toolkit [GATK]; Broad Institute, Cambridge, MA, USA) was used for single nucleotide polymorphism (SNP) and indel calling. Visualization and filtration of the sequence variations were performed using a high-performance viewer (Integrative Genomics Viewer [IGV]; Broad Institute) in comparison with SNP information (common, flagged, and multiple-location mapped SNPs) from the dbSNP137 database.

Identification of Variants

Due to the overlap of clinical features and RP-associated genes with other inherited retinal diseases, 86 genes associated with RP, Leber congenital amaurosis (LCA), and cone-rod dystrophy (CRD) were analyzed for variant detection (Supplementary Fig. S1).

Filtration and Prioritization of Variants

To distinguish between pathogenic and nonpathogenic variants, the annotated sequencing data were filtered and prioritized based on the following criteria: (1) variant located in coding sequence; (2) nonsynonymous variant; (3) nonsense variant; (4) short insertion or deletion variant (indel) in coding sequence; (5) variant located at splice donor or acceptor site, and (6) variant reported in flagged SNPs but not in common or multiple-location mapped SNPs of the dbSNP137 database.

In Silico Prediction of Variants

Six computational tools were used to predict the possible effects of the identified amino acid substitutions on protein function: PolyPhen-2, (SIFT), VarioWatch, MutationTaster, Prediction of Pathological Mutations (PMut), and Screening for Non-Acceptable Polymorphisms (SNAP). Only the following outputs were considered as deleterious variants: PolyPhen-2 (probably or possibly damaging); SIFT (damaging); VarioWatch (very high or high); MutationTaster (disease causing); PMut (pathological); and SNAP (non-neutral). For estimating evolutionary conservation, multiple sequence alignments of the affected amino acids in different vertebrate species were performed using sequence alignment program (ClustalW; The Biology Workbench, San Diego, CA, USA).

Variant Validation

The workflow for variant validation is shown in Supplementary Figure S2. Variants were selected for confirmation by Sanger sequencing when three or more of the prediction outputs from the six computational prediction tools suggested that a variant was deleterious. The primer sequencing program (Primer3; University of Massachusetts, Boston, MA, USA) was used for designing amplicon-specific primers to amplify the regions containing each variant. The PCR products were treated with a reagent (ExoSAP-IT; Affymetrix, Santa Clara, CA, USA) before processing for direct sequencing. Sequencing was performed using a cycle-sequencing kits (ABI BigDye Terminator v3.1; Applied Biosystems, Grand Island, NY, USA) on an analyzer (ABI 3130xl Genetic Analyzer; Applied Biosystems). To verify the pathogenicity of novel variants identified in this study, denaturing HPLC (Transgenomic, Inc., San Jose, CA, USA) analysis was performed for detecting each variant on at least 110 DNA samples (220 chromosomes) from unrelated healthy individuals.

Segregation Analysis

To assess variant segregation within each patient's family, Sanger sequencing was also performed on the family members from whom DNA was available.

Protein Structure and Functional Prediction

The protein structures and functions resulting from missense variants identified in this study were predicted using the freely available web service HOPE (Have Your Protein Explained).[18]

Results

Twenty unrelated Thai patients were recruited for this pilot study. Most patients experienced poor night vision. The BCVA ranged from hand motion (HM) to 6/6. The OCT was performed in two patients (RP023 and RP069), which demonstrated no evidence of cystoid macular edema. A summary of clinical information and family pedigrees are

Table 1. Clinical Information for Patients in this Study

		Age			BCVA		ERG		VEP		
Sample	Sex	Onset	At Exam	Presenting Symptoms	RE	LE	RE	LE	RE	LE	Fundus Examination
RP009	M	27	30	Poor night vision; flashing	6/6	6/9	NR	NR	Normal	Normal	Pink optic disc; generalized RPE changes without macular involvement; moderate bone spicules
RP010	F	60	69	Progressive visual loss	6/24	NLP	NR	NR	Normal	NR	Pale optic disc LE > RE; heavy bone spicules 360 degrees
RP011	F	19	29	Poor night vision	6/9	6/9	NR	NR	Normal	Normal	Mildly pale optic disc; generalized RPE changes without macular involvement; mild bone spicules
RP016	M	26	36	Progressive visual loss; poor night vision	CF	CF	NR	NR	Moderately decreased amplitude	Moderately decreased amplitude	Pale optic disc; generalized RPE changes; macular hyperpigmentation; bone spicules in macula only
RP019	F	47	49	Progressive visual loss; poor night vision; poor color discrimination	6/24	6/18	NR	NR	Normal	Normal	Pale optic disc; moderate bone spicules in mid periphery
RP022	F	11	11	Blurred vision since birth; photophobia; poor night vision	6/60	6/60	NR	NR	Normal	Normal	Pale optic disc; heavy bone spicules 360 degree; macular RPE changes
RP023*	M	25	33	Progressive visual loss; poor night vision; tearing; photosensitivity	6/60	6/18	NR	NR	Moderately decreased amplitude	Moderately decreased amplitude	Pale optic disc; heavy bone spicules 360 degrees; macular RPE changes
RP027	M	50	51	Poor night vision	6/36	6/24	NR	NR	Moderately decreased amplitude	Moderately decreased amplitude	Pink optic disc; RPE changes in midperiphery without macular involvement; no bone spicules
RP038	M	18	48	Progressive visual loss; flashing	HM	CF	NR	NR	Markedly decreased amplitude	Markedly decreased amplitude	Pale optic disc; generalized RPE changes with macular involvement; minimal bone spicules
RP069*	M	19	23	Progressive visual loss; poor night vision	HM	HM	NR	NR	Markedly decreased amplitude	Markedly decreased amplitude	Pale optic disc; generalized RPE changes with macular sheen; no bone spicules
RP087/1*	M	3	5	Blurred distant vision; poor night vision; high myopia	6/36	6/60	NR	NR	Normal	Normal	Pink disc; generalized RPE changes without macular involvement; no bone spicules
RP087/2*	M	5	10	Blurred distant vision; poor night vision; mild myopia with high astigmatism	6/60	6/60	NR	NR	Normal	Normal	Pink disc; generalized RPE changes without macular involvement; no bone spicules

RE, right eye; LE, left eye; CF, counting fingers; VF, visual field; NR, nonrecordable; NLP, no light perception due to anterior ischemic optic neuropathy.
* Consanguineous family.

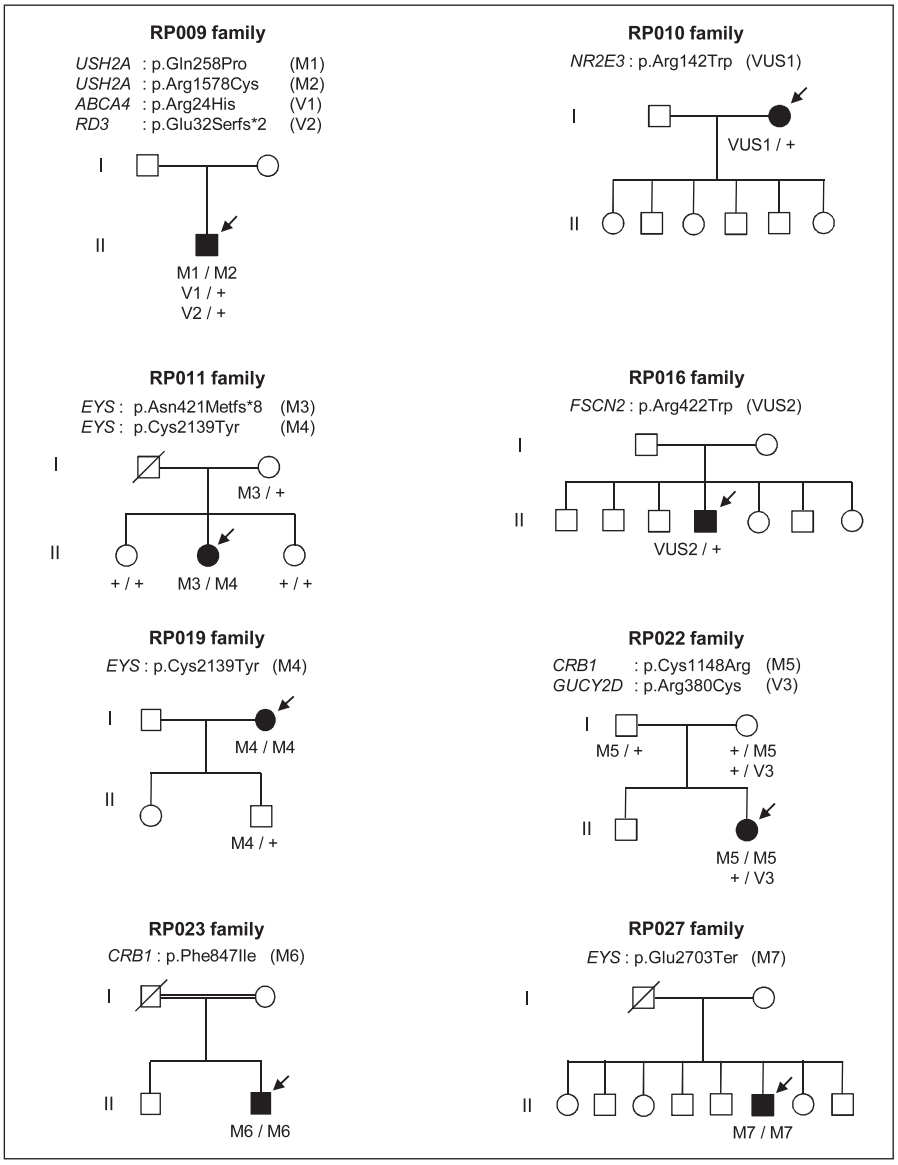

FIGURE. Pedigrees and genotypes of the RP families. Genotype data are presented below the patient and members, where applicable, of their family. *Filled symbols with an arrow* indicate the probands. *Squares*: males. *Circles*: females. *Slashed*: decreased family members. *Double line*: consanguineous marriage. *Filled black circle*: obligate carrier. Normal alleles are indicated by '+'. Potentially pathogenic mutations, additional variants, and variants of uncertain significance, are indicated by "M," "V," and "VUS," respectively.

shown in Table 1 and the Figure, respectively. The average read depth of WES observed in the 20 samples was approximately 150. The putative deleterious variants were detected in 11 out of 20 patients (55%). Nine of them carried a total of 10 potentially pathogenic mutations and five additional heterozygous variants, while the other two having two variants of uncertain significance (VUS).

Potentially Pathogenic Mutations

Ten different putative, disease-causing mutations, eight novel and two known, were identified in nine patients (Table 2, Supplementary Table S1). These include five autosomal recessive genes (*CRB1*, *C8orf37*, *EYS*, *PROM1*, and *USH2A*) and one X-linked recessive gene (*RP2*). These mutations include five missense substitutions, two nonsense mutations, two deletions, and one splice site change. None of the novel missense variants were detected in at least 220 alleles from the unrelated normal controls.

Additional Heterozygous Variants

Among the nine patients with potentially pathogenic mutations, three (RP009, RP022, and RP038 probands) also carried a heterozygous variant in one of five genes (*ABCA4*, *GUCY2D*, *RD3*, *ROM1*, and *TULP1*; Fig., Table 2).

The RP009 proband carried four variants in three genes (*USH2A*, *ABCA4*, and *RD3*) (Fig., Tables 2, 3, Supplementary Fig. S3). A compound heterozygous mutation of a known variant, c.4732C>T (p.Arg1578Cys), and a novel variant, c.773A>C (p.Gln258Pro), were identified in *USH2A*. The variant p.Arg1578Cys was previously reported as a disease-causing mutation in a patient with Usher syndrome type 2 (USH2),[19] while the p.Gln258Pro variant was not detected in the screening of 224 normal chromosomes. For the *ABCA4* gene, a single known heterozygous mutation reported in a patient with Stargardt disease type 1,[20] c.71G>A (p.Arg24His), was detected. Finally, a novel heterozygous frameshift variant, c.94_95delG (p.Glu32Serfs*2), was found in the *RD3* gene.

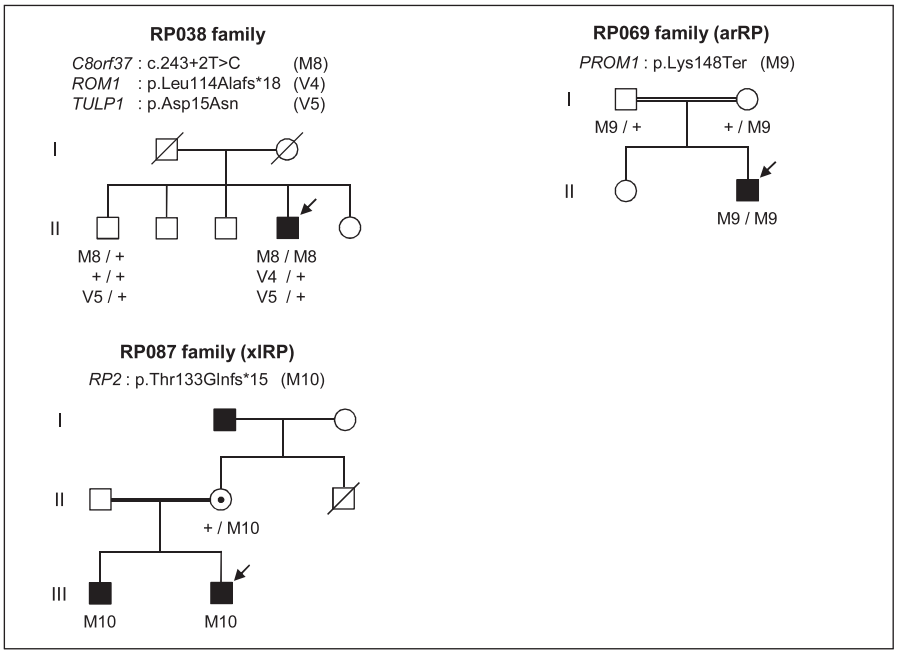

Figure. Continued.

In addition, the RP022 proband carried two variants, but three alleles, in genes *CRB1* and *GUCY2D* (Fig.). In the *CRB1* gene, a novel homozygous mutation, c.3442T>C (p.Cys1148Arg), was detected. The occurrence of this mutation at a highly conserved cysteine (Supplementary Fig. S3), its absence in the 242 normal chromosomes screened, and the results of the six prediction tools support its true pathogenicity (Table 3). In addition, HOPE prediction showed that the substitution from cysteine 1148 to arginine changed a highly conserved cysteine in the EGF-like 15 domain and caused a disruption of the disulfide bridge with Cys1163, which in turn could lead to misfolding and thereby affect the binding properties of *CRB1* product (crumbs homolog 1). Both asymptomatic parents and a brother of the patient were found to be heterozygous carriers of p.Cys1148Arg. Another novel heterozygous variant in the *GUCY2D* gene, c.1138C>T (p.Arg380Cys), was detected in the patient and his unaffected mother.

Variants of Uncertain Significance (VUS)

Single novel heterozygous variants predicted to be deleterious were detected in genes *FSCN2* and *NR2E3* in two isolated RP patients (Fig., Table 2). These include a c.1264C>T (p.Arg422Trp) variant in *FSCN2* and a c.424C>T (p.Arg142Trp) variant in the *NR2E3* identified in the RP016 and RP010 probands, respectively.

Discussion

We present here the first mutation report in Thai patients with nonsyndromic RP using WES. Eighty-six genes associated with RP, LCA, and CRD were analyzed to identify potentially pathogenic variants. Our study in 20 unrelated patients uncovered 10 potentially pathogenic mutations and two VUSs in nine and two patients, respectively. We also describe genotype–phenotype correlations of the identified genetic variants (Tables 1, 2).

Potentially Pathogenic Mutations With and Without the Additional Variants

The RP038 proband carries three variants, but four alleles in three genes, *C8orf37*, *ROM1*, and *TULP1*. A novel homozygous splice-donor-site mutation, c.243+2T>C, identified in *C8orf37* is most likely to be the cause of arRP in this family. This change is predicted to introduce a premature termination codon (PTC) that could lead to activation of the nonsense-mediated mRNA decay (NMD) pathway.[21] The proband's phenotype was accordant with a previous report in which all affected members demonstrated early impairment of the macula with age of onset ranging from infancy to late teenage years.[13] Furthermore, two additional variants were identified in the *ROM1* and *TULP1* genes. A frameshift insertion in *ROM1*, c.331_332insG (dbSNP rs137955062), was previously described in monogenic adRP[22,23] with disease severity ranging from asymptomatic to severe, and in digenic RP when coinherited with a p.Leu185Pro mutation in the *PRPH2* gene.[24] This variant was also detected in two out of 240 normal chromosomes, indicating that the c.331_332insG variant is not the causative mutation for the RP038 proband. A novel heterozygous variant, c.43G>A, in *TULP1* was identified in the RP038 proband and his unaffected elder brother. Previous reports have described *TULP1* variants as the causes of both arRP and arLCA (RetNet). These data imply that the change in *TULP1* in the RP038 proband does not correlate with the disease.

In our study, two *CRB1* gene mutations in exons 9 (RP022 proband) and 7 (RP023 proband) were identified. This is in accordance with previous publications that showed mutations mainly in exons 9 (41%) and 7 (27%).[25] These findings confirm the importance of the domains included in these two exons, exon 7: laminin G-like 2, exon 9: laminin G-like 3 and EGF-like 15 through 17. Previous reports of *CRB1* gene mutations in retinal dystrophies showed variable age of onset and BCVA.[25,26] In the RP022 proband, who showed onset of blurred vision since birth, a homozygous mutation (p.Cys1148Arg) in *CRB1* and a heterozygous variant (p.Arg380Cys) in *GUCY2D* were detected, whereas a homozygous mutation (p.Phe847Ile) in

Table 2. Variants Identified in the RP Patients

Sample	MOI in Family	Gene	MOI of Gene	Variant Detected						Reference
				Chr	Exon	Nucleotide Change	Amino Acid Change	State	Domain	
Potentially pathogenic mutations										
RP009	Isolated RP	*USH2A*	arRP	1	4	c.773A>C	p.Gln258Pro	Het	–	Novel
		USH2A		1	22	c.4732C>T	p.Arg1578Cys	Het	Laminin G-like 1	Reported[19]
RP011	Isolated RP	*EYS*	arRP	6	8	c.1260_1260delG	p.Asn421Metfs*8	Het	–	Novel
		EYS		6	31	c.6416G>A	p.Cys2139Tyr	Het	EGF-like 21	Reported[31]
RP019	Isolated RP	*EYS*	arRP	6	31	c.6416G>A	p.Cys2139Tyr	Hom	EGF-like 21	Reported[31]
RP022	Isolated RP	*CRB1*	arRP and arLCA	1	9	c.3442T>C	p.Cys1148Arg	Hom	EGF-like 15	Novel
RP023	arRP	*CRB1*	arRP and arLCA	1	7	c.2539T>A	p.Phe847Ile	Hom	Laminin G-like 2	Novel
RP027	Isolated RP	*EYS*	arRP	6	42	c.8107G>T	p.Glu2703Ter	Hom	–	Novel
RP038	Isolated RP	*C8orf37*	arRP and arCRD	8	Intron 2	c.243+2T>C	splice site change	Hom	–	Novel
RP069	arRP	*PROM1*	arRP and adCRD	4	4	c.442A>T	p.Lys148Ter	Hom	Cytoplasmic	Novel
RP087	xlRP	*RP2*	xlRP	X	2	c.395_419delCCACTCAA CCCATCATTGAGTCTTC	p.Thr133Glnfs*15	Hem	C-CAP/cofactor C-like	Novel
Additional heterozygous variants										
RP009	Isolated RP	*ABCA4*	arRP and arCRD	1	2	c.71G>A	p.Arg24His	Het	Transmembrane	Reported[20]
		RD3	arLCA	1	2	c.94_95delG	p.Glu32Serfs*2	Het	Coiled coil	Novel
RP022	Isolated RP	*GUCY2D*	adCRD and arLCA	17	4	c.1138C>T	p.Arg380Cys	Het	Extracellular	Novel
RP038	Isolated RP	*ROM1*	adRP	11	1	c.339_340insG	p.Leu114Alafs*18	Het	Transmembrane	Reported[22–24]
		TULP1	arRP and arLCA	6	1	c.43G>A	p.Asp15Asn	Het	–	Novel
Variants of uncertain significance										
RP010	Isolated RP	*NR2E3*	arRP and adRP	15	4	c.424C>T	p.Arg142Trp	Het	–	Novel
RP016	Isolated RP	*FSCN2*	adRP	17	4	c.1264C>T	p.Arg422Trp	Het	Fascin-2	Novel

Nucleotide numbering reflects cDNA numbering with position 1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (provided in the public domain by The Human Genome Variation Society, http://www.hgvs.org/mutnomen). The initiation codon is codon 1. The nucleotide position of each gene was based on these GenBank cDNAs (accession number): *ABCA4* (NM_000350.2), *C8orf37* (NM_177965), *CRB1* (NM_201253.2), *EYS* (NM_001142800.1), *FSCN2* (NM_012418.3), *GUCY2D* (NM_000180.3), *NR2E2* (NM_014249.2), *PROM1* (NM_006017.2), *RD3* (NM_001164688.1), *ROM1* (NM_000327.3), *RP2* (NM_006915.2), *TULP1* (NM_003322.3), and *USH2A* (NM_206933.2). MOI, mode of inheritance; Het, heterozygous; Hom, homozygous; Hem, hemizygous.

Table 3. Summary of Variant Prediction Using Six Different Tools

Sample	Gene	Amino Acid Change	Prediction Tool*						Familial Segregation	Alleles in Control† Chromosomes	Vertebrate Conservation‡
			PolyPhen-2	SIFT	VarioWatch	MutationTaster	PMut	SNAP			
Potentially pathogenic mutations											
RP009	*USH2A*	p.Gln258Pro	Probably damaging	Damaging	High	Disease causing	Neutral	Non-neutral	NA	0/224	Yes
	USH2A	p.Arg1578Cys	Possibly damaging	Damaging	High	Disease causing	Neutral	Non-neutral	NA	NA§	Yes
RP011	*EYS*	p.Asn421Metfs*8	–	–	–	Disease causing	–	–	Yes	NA	Yes
	EYS	p.Cys2139Tyr	Probably damaging	Damaging	High	Polymorphism	Pathological	Non-neutral	Op	NA§	Yes
RP019	*EYS*	p.Cys2139Tyr	Probably damaging	Damaging	High	Polymorphism	Pathological	Non-neutral	Yes	NA§	Yes
RP022	*CRB1*	p.Cys1148Arg	Probably damaging	Damaging	High	Disease causing	Pathological	Non-neutral	Yes	0/242	Yes
RP023	*CRB1*	p.Phe847Ile	Possibly damaging	Tolerated	High	Polymorphism	Neutral	Non-neutral	NA	0/224	Yes
RP027	*EYS*	p.Glu2703Ter	–	–	Very high	Disease causing	–	–	NA	NA	Yes
RP038	*C8orf37*	Splice site change	–	–	–	Disease causing	–	–	Yes	NA	–
RP069	*PROM1*	p.Lys148Ter	–	–	Very high	Disease causing	–	–	Yes	NA	Yes
RP087	*RP2*	p.Thr133Glnfs*15	–	–	–	Disease causing	–	–	Yes	NA	Yes
Additional heterozygous variants											
RP009	*ABCA4*	p.Arg24His	Probably damaging	Tolerated	High	Disease causing	Pathological	Non-neutral		NA§	Yes
	RD3	p.Glu32Serfs*2	–	–	–	Disease causing	–	–	–	NA	Yes
RP022	*GUCY2D*	p.Arg380Cys	Probably damaging	Damaging	High	Disease causing	Neutral	Neutral	Opo	0/220	Yes
RP038	*ROM1*	p.Leu114Alafs*18	–	–	–	Disease causing	–	–	Op	2/240	Yes
	TULP1	p.Asp15Asn	Probably damaging	Tolerated	High	Polymorphism	Neutral	Non-neutral	Opo	0/232	Yes
Variants of uncertain significance											
RP010	*NR2E3*	p.Arg142Trp	Possibly damaging	NA	NA	Disease causing	Pathological	Non-neutral	NA	0/236	Yes
RP016	*FSCN2*	p.Arg422Trp	Possibly damaging	Tolerated	High	Polymorphism	Pathological	Non-neutral	NA	0/224	No

NA, not available; Op, observed in proband only; Opo, observed in proband and other(s).

* Output prediction of each tool—PolyPhen-2 classification: possibly damaging, probably damaging, benign. SIFT classification: damaging, tolerated. MutationTaster classification: disease-causing, polymorphism. VarioWatch classification: very high, high, medium, low risk levels. PMut classification: pathological, neutral. SNAP: non-neutral, neutral.

† Control—Allele frequencies were measured in at least 110 controls (220 alleles) from unrelated normal controls.

‡ Vertebrate conservation—Yes, conserved in more than 80% of all vertebrates aligned; No, conserved in less than 80% of all vertebrates aligned.

§ Known variant.

CRB1 was identified in the RP023 proband with onset of RP occurring at age 25 years. Given the fact that various *GUCY2D* gene mutations have been reported to cause both adCRD and arLCA (RetNet), and that the p.Arg380Cys variant was predicted to be deleterious, *GUCY2D* cannot be excluded from acting as a possible modifier gene. Coinheritance of variants in both *CRB1* and *GUCY2D* might explain the earlier onset of the disease in the RP022 proband compared with the RP023 proband.

In a previous report, a compound heterozygous and a homozygous mutation identified in the *USH2A* gene were associated with USH2 and RP without hearing loss, respectively.[27] Here, four variants in three different genes (*USH2A*, *ABCA4*, and *RD3*) were detected in the RP009 proband. Among the identified variants, a compound heterozygous mutation (p.Arg1578Cys and p.Gln258Pro) detected in USH2A is likely the cause of the disease. While the p.Arg1578Cys mutation was previously reported in a patient with USH2,[19] the RP009 proband has no hearing loss at age 30 years. Previous reports showed a wide range of BCVA among patients with *USH2A* mutations.[28,29] The BCVA and the macula of the RP009 proband were both normal. Although single heterozygous *ABCA4* gene variants have been reported to cause age-related macular degeneration,[4,30] this finding was not observed in the RP009 proband at the time of examination. For *RD3*, all causative mutations were reported only in arLCA. Due to the lack of cosegregation analysis in family members, it is difficult to describe the *ABCA4* and *RD3* gene variants as possible modifier genes or as possible causes of phenotypic variation within the patient's family.

Reported *EYS* mutations are distributed along the length of the gene.[31,32] In this study, a reported homozygous missense mutation (p.Cys2139Tyr) in exon 31[31] and a novel homozygous nonsense mutation (p.Glu2703Ter) in exon 42 of *EYS* were detected in the RP019 and RP027 probands, respectively. Normally, the Cys residue at position 2139 forms a disulfide bridge with Cys2130 (provided in the public domain by The Universal Protein Resource [UniProt], http://www.uniprot.org/uniprot/Q5T1H1/). Substitution of p.Cys2139Tyr may affect the conformation of the EYS protein. In the RP011 proband, a compound heterozygous mutation of a novel frameshift mutation (p.Asn421Metfs*8) in exon 8 and the same missense mutation as in the RP019 proband were observed (Table 2). The p.Glu2703Ter mutation is located near the C-terminal, leading to the loss of 441 amino acids in the EYS protein, while the p.Asn421Metfs*8 mutation introduces eight new amino acids. Both null alleles are predicted to induce a PTC that can lead to activation of the NMD pathway.[21] The RP011 proband developed poor night vision at age 19. This data supports the previous report in a French family with a compound heterozygous mutation of p.Cys2139Tyr and c.2847-1G>T with RP onset at age 21 years.[31] A homozygous nonsense mutation (p.Glu1836Ter) in exon 26 reported in a Chinese arRP patient was associated with onset of night blindness at age 15 years,[33] while the RP027 proband with the mutation in exon 42 developed poor night vision at age 50 years. The severity of protein truncation may determine the difference in age of onset.

In the RP069 proband, a novel homozygous nonsense mutation (p.Lys148Ter) in the *PROM1* gene leads to a truncation of 717 C-terminal amino acids from the full-length 865 residues and could activate the NMD pathway.[21] Homozygous truncation mutations in *PROM1* have been identified in CRD and RP patients with early onset and severe visual impairment (6/120 to HM).[34–37] Our patient developed progressive visual loss since age 19 years, which was later than those reported previously. However, the BCVA rapidly deteriorated to HM at age 23 years. The severity of visual impairment can be explained by an in vivo study that showed expression of *PROM1* by rod and cone photoreceptor cells. The absence of *PROM1* product (prominin-1 protein) specifically impaired photoreceptor outer segment and disk morphogenesis.[38]

A novel 25-bp deletion in exon 2 of the *RP2* gene, identified in the RP087 family, is located in the C-CAP/cofactor C-like domain. This frameshift deletion leads to a truncated XRP2 protein missing 217 C-terminal amino acids from the full-length 350 residues possibly resulting in activation of the NMD pathway.[21] Exon 2 is the most common site of mutations reported in HGMD (provided in the public domain by The Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php), suggesting the importance of this domain on XRP2 protein function. The proband RP087/1 and his affected elder brother, RP087/2, developed visual impairment at the ages of three and five, respectively. High myopia and high astigmatism were demonstrated. This information supports the genotype–phenotype correlation of mutations in *RP2* with early onset of the disease and a high degree of refractive error.[39,40]

Variants of Uncertain Significance

A novel missense variant (p.Arg422Trp) in the *FSCN2* gene identified in the RP016 proband is associated with disease onset at age 26 and severe visual impairment. Only one reported mutation, 208delG, has been identified in Japanese families with adRP and autosomal dominant macular dystrophy (adMD).[41,42] However, this mutation was also identified in both affected and unaffected members of Chinese families with RP, CRD, LCA, and normal controls, implying that 208delG was not the causative mutation of these diseases.[43] Different variants in *FSCN2* were detected in Spanish patients with adRP and adMD, but none of these variants cosegregated in the families.[44] The *FSCN2* gene product (retinal fascin 2) was proposed to play a role in photoreceptor disk morphogenesis.[45] Although the arginine residue 422 of retinal fascin 2 is not evolutionarily conserved, the results from HOPE prediction showed that Arg422 is located on the surface of this protein. The hydrophobicity difference between the arginine and tryptophan residues may affect the hydrogen-bond formation leading to the loss of protein interactions with other molecules. In fact, no tryptophan homologues of *FSCN2* were found during evolutionary analysis as assessed by multiple alignments among distant species. This residue is normally glutamine in other vertebrates (Supplementary Fig. S3). A lack of DNA samples from additional family members makes it impossible to describe the segregation of p.Arg422Trp in this family. Cosegregation analysis and further in-depth functional study of this variant is needed for understanding the disease mechanism.

A novel heterozygous variant (p.Arg142Trp) in the *NR2E3* gene was identified in the RP010 proband. A previous study showed 33 disease-causing *NR2E3* mutations with only one mutation causing adRP and two mutations causing arRP.[46] The RP010 proband demonstrated a relatively good prognosis with late disease onset and 6/24 vision in her right eye. Her left eye had no light perception due to anterior ischemic optic neuropathy. Although the identified variant (p.Arg142Trp) was predicted to be deleterious, it would require the family's DNA samples to confirm the significance of this missense variant.

In summary, WES is a powerful approach for the identification of pathogenic genes in RP. Further analysis will reveal other known or novel genes contributing to the disease.

Web Resources

The URLs for data presented herein are provided in the public domain as follows:

1. RetNet, https://sph.uth.edu/RetNet/

2. BWA, http://bio-bwa.sourceforge.net/
3. SAMtool, http://samtools.sourceforge.net/
4. GATK, http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit
5. Integrative Genomics Viewer (IGV), http://www.broadinstitute.org/igv
6. Polymorphism Phenotyping v2 (PolyPhen-2), http://genetics.bwh.harvard.edu/pph2/index.shtml
7. Sorting Intolerant From Tolerant (SIFT), http://sift.jcvi.org/www/SIFT_enst_submit.html
8. VarioWatch, http://genepipe.ncgm.sinica.edu.tw/variowatch/main.do
9. MutationTaster, http://www.mutationtaster.org/
10. PMut, http://mmb2.pcb.ub.es:8080/PMut/
11. SNAP, https://rostlab.org/services/snap/
12. HOPE, http://www.cmbi.ru.nl/hope/home
13. ClustalW, http://workbench.sdsc.edu/
14. Primer3 program, http://simgene.com/Primer3

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