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RPE65 mutation frequency and phenotypic variation according to exome sequencing in a tertiary centre for genetic eye diseases in China

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ABSTRACT.

Purpose: **Retinoid isomerohydrolase RPE65 has received a tremendous amount of attention due to successful clinical gene therapy for Leber congenital amaurosis (LCA) cases caused by *RPE65* mutations. This study aimed to evaluate the frequency of *RPE65* mutations and the associated phenotypes based on exome sequencing.**

Methods: ***RPE65*** **variants were collected from exome sequencing data obtained from 2133 probands with different forms of hereditary retinal degeneration (HRD). Clinical data were collected from probands with homozygous or compound heterozygous variants in *RPE65*. Associated phenotypes were characterized based on clinical data.**

Results: **Biallelic *RPE65* mutations were detected in 18 families, including eight with LCA, five with early-onset retinal degeneration, four with fundus albipunctatus-like (FA-like) changes and one with high hyperopia. These cases accounted for approximately 3.0% (8/269) of LCA and 0.8% (18/2133) of HRD cases. An almost identical FA-like change was identified in seven patients from four unrelated families with *RPE65* mutations. Classification of mutations suggested that FA-like changes may be associated with biallelic missense mutations in *RPE65*.**

Conclusion: **Fundus albipunctatus-like (FA-like) change, a common characteristic fundus sign in *RPE65* biallelic mutations, was unexpected but was confirmed by the finding that affected siblings from different families exhibited similar phenotypes. These results enrich our understanding of *RPE65* mutation frequencies and their associated phenotypic variants.**

Key words: fundus albipunctatus – mutation frequency – phenotype – *RPE65*

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Introduction

Retinoid isomerohydrolase RPE65, an enzyme encoded by the *RPE65* gene (HGNC ID: 10294, OMIM: 180069) (Nicoletti et al. 1995), is responsible for the conversion of all-trans-retinyl esters to 11-cis-retinol during visual phototransduction in the retinal pigment epithelium (Xue et al. 2004; Moiseyev et al. 2005). Biallelic mutations in *RPE65* are an important cause of Leber's congenital amaurosis (LCA) (Marlhens et al. 1997; den Hollander et al. 2008; Cideciyan 2010), the most severe form of hereditary retinal degeneration (HRD) that causes individuals to be born blind. *RPE65*-associated LCA has gained a great deal of public attention as a result of a clinical gene therapy (Lee & Lotery 2017; Apte 2018; Kumaran et al. 2018); this new gene therapy targets LCA caused by *RPE65* mutations and was recently approved by the Food and Drug Administration as the first directly administered gene therapy of its kind (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm589467.htm).

The identification of patients with *RPE65* mutations is a prerequisite for developing *RPE65*-targeted gene therapies. Patients with *RPE65* mutations frequently manifest as LCA (Weleber et al. 1993; Gu et al. 1997; Morimura et al. 1998; Cideciyan 2010), occasionally present as early-onset HRD or retinitis pigmentosa (Gu et al. 1997; Morimura et al. 1998) and rarely present as fundus albipunctatus (FA) (Schatz et al. 2011; Hull et al. 2016; Yang et al. 2017) or cone-rod dystrophy (Jakobsson et al. 2014). *RPE65* mutations are thought to be responsible for approximately 6% of all LCA cases in Caucasians (den Hollander et al. 2008) but for only a few LCA cases in Chinese populations (Xu et al. 2016). However, most of these results are based on analyses of subgroups of patients that consist mostly of those with LCA. Neither the frequency of *RPE65* mutations in all forms of HRD nor the variety of associated phenotypes has been well studied. As

Table 1. Rare variants in biallelic status in 18 probands with genetic eye diseases.

Variant	Exon	Position at chr01	Nuleotide change	Effect	Poly Phen-2 Score	SIFT Score	Mutation taster score	CADD score	Berkeley Drosophila Genome Project	Human Splicing Finder	Exome Aggregation Consortium allele frequency			1000 genome		Human Genome Mutation Database	rs ID	First reported
											All	East Asian	Homo	All	South Han			
1	2	68914307	c.94G>T	p.Gly32Cys	PD (0.985)	D (0.000)	DC (1.000)	33.0	/	/	1/118428	0/8592	0	NA	NA	DM	NA	Astuti et al. (2016)
2	3	68912507	c.131G>A	p.Arg44Gln	PD (1.000)	D (0.000)	DC (1.000)	32.0	/	/	7/121406	0/8654	0	0.0002	0.0000	DM	rs61751282	Simovich et al. (2001)
3	3	68912448	c.190C>G	p.Gln64Glu	PD (0.996)	D (0.000)	DC (1.000)	25.8	/	/	NA	NA	NA	NA	NA	NA	NA	Novel
4	3	68912438	c.200T>G	p.Leu67Arg	PD (1.000)	D (0.000)	DC (1.000)	28.7	/	/	NA	NA	NA	NA	NA	DM	NA	Xu et al. (2012)
5	4	68910541	c.271C>T	p.Arg91Trp	PD (0.999)	D (0.010)	DC (1.000)	27.4	/	/	10/120738	1/8620	0	0.0002	0.0000	DM	rs61752871	Morimura et al. (1998)
6	4	68910517	c.295G>A	p.Val99Ile	B (0.077)	D (0.020)	DC (1.000)	23.8	/	/	36/120964	31/8632	1	0.0012	0.0048	NA	rs143056561	Li et al. (2011)
7	5	68910345	c.364T>C	p.Tyr122His	B (0.046)	T (0.120)	DC (1.000)	23.0	/	/	NA	NA	NA	NA	NA	NA	NA	Novel
8	5	68910279	c.430T>C	p.Tyr144His	PD (1.000)	D (0.030)	DC (1.000)	27.4	/	/	NA	NA	NA	NA	NA	DM	NA	Chen et al. (2013)
9	5	68910275	c.434C>A	p.Ala145Asp	PD (0.999)	D (0.000)	DC (1.000)	26.8	/	/	NA	NA	NA	NA	NA	DM	NA	Fu et al. (2013)
10	5	68910216	c.493C>T	p.Gln165*	/	/	DC (1.000)	41.0	/	/	NA	NA	NA	NA	NA	DM	NA	Wang et al. (2015)
11	6	68906634	c.545A>G	p.His182Arg	PD (0.993)	D (0.000)	DC (1.000)	25.4	/	/	NA	NA	NA	NA	NA	DM	NA	Jacobson et al. (2005)
12	6	68906540	c.639dupA	p.Ala214 Serfs*20	/	/	DC (1.000)	/	/	/	NA	NA	NA	NA	NA	DM	NA	Wang et al. (2016)
13	7	68905256	c.713C>G	p.Ser238Cys	PD (1.000)	D (0.000)	DC (1.000)	29.9	/	/	NA	NA	NA	NA	NA	NA	NA	Novel
14	7	68905247	c.722A>G	p.His241Arg	PD (1.000)	D (0.000)	DC (1.000)	25.9	/	/	NA	NA	NA	NA	NA	NA	NA	Novel
15	8	68904907	c.825C>A	p.Tyr275*	/	/	DC (1.000)	38.0	/	/	NA	NA	NA	NA	NA	NA	NA	Novel
16	9	68904626	c.997G>C	p.Gly333Arg	PD (1.000)	D (0.010)	DC (1.000)	34.0	/	/	NA	NA	NA	NA	NA	NA	NA	Li et al. (2011)
17	Intron 9	68904624	c.998+1G>A	SD	/	/	DC (1.000)	33.0	SSC	SSC	NA	NA	NA	NA	NA	NA	NA	Novel
18	Intron 9	68904000	c.999-1G>T	SA	/	/	DC (1.000)	34.0	SSC	SSC	NA	NA	NA	NA	NA	NA	NA	Novel
19	10	68903939	c.1059dupG	p.Lys354 Glufs*11	/	/	DC (1.000)	35.0	/	/	NA	NA	NA	NA	NA	DM	NA	Jacobson et al. (2007)
20	10	68903931	c.1067delA	p.Asn356 Metfs*17	/	/	DC (1.000)	34.0	/	/	NA	NA	NA	NA	NA	DM	rs281865520	Marlhens et al. (1997)
21	12	68897002	C.1301C>A	p.Ala434Glu	PD (0.999)	T (0.650)	DC (1.000)	23.7	/	/	NA	NA	0	NA	NA	NA	NA	Novel
22	13	68896824	c.1374G>A	p.Trp458*	/	/	DC (1.000)	43.0	/	/	NA	NA	NA	NA	NA	DM	NA	Astuti et al. (2016)
23	13	68896799	c.1399C>G	p.Pro 467Ala	PB (0.918)	D (0.050)	DC (1.000)	25.7	/	/	NA	NA	NA	NA	NA	NA	NA	Novel
24	Intron 13	68896747	c.1450+1delG	SD	/	/	DC (1.000)	/	SSC	SSC	NA	NA	NA	NA	NA	NA	NA	Novel

Table 1. (Continued)

Variant	Exon	Position at chr01	Nucleotide change	Effect	Poly Phen-2 Score	SIFT Score	Mutation taster score	CADD score	Berkeley Drosophila Genome Project	Human Splicing Finder	Exome Aggregation Consortium allele frequency			1000 genome		Human Genome Mutation Database	rs ID	First reported
											All	East Asian	Homo	All	South Han			
25	Intron 13	68895611	c.1451-1G>A	SA	/	/	DC (1.000)	34.0	SSC	SSC	NA	NA	NA	NA	NA	NA	NA	Novel
26	14	68895558	c.1503T>G	p.Tyr501*	/	/	DC (1.000)	36.0	/	/	NA	NA	NA	NA	NA	NA	NA	Novel
27	14	68895518	c.1543C>T	p.Arg 515Trp	PD (1.000)	D (0.000)	DC (1.000)	27.7	/	/	2/120986	0/8624	0	NA	NA	DM	rs121917745	Kondo et al. (2004)

A CADD score over 20 suggests potential damaging.
B = benign, D = damaging, DC = disease causing, PB = possibly damaging, PD = probably damaging, SA = splicing acceptor, SD = splicing donor, SSC = splice site changed, T = tolerated.

one disease may mimic another due to phenotypic variation or differences among the stages of the diseases, it is important to determine whether other forms of retinal degeneration are caused by *RPE65* mutations. Genotype-guided phenotype characterization may provide a different view of this than can be achieved by other approaches, such as phenotype-guided genotype analysis performed in one or a set of candidate genes.

Whole exome sequencing (WES) and targeting exome sequencing (TES) provide genotype information for a group of genes in individuals with different phenotypes. These data provide a practical platform for performing genotype-guided phenotypic characterization (to determine to what extent the phenotype may vary) of genes in patients with different forms of related diseases have been analysed by WES. In the current study, sequencing variants of *RPE65* were selected from our in-house data obtained from 2133 probands with suspected HRD who underwent WES or TES analysis. Biallelic potential pathogenic mutations were identified in 18 families with phenotypes associated with LCA, early-onset retinal degeneration (EORD), FA-like (FA-like) change or high hyperopia. Two of the 18 families with LCA were previously reported and also included in the current analysis, in which *RPE65* mutations were detected by WES or Sanger sequencing. These results provide a brief overview of *RPE65* mutation frequencies in addition to data indicating the phenotypic variation that is associated with *RPE65* mutations.

Materials and Methods

Probands and family members

Data obtained from probands with different forms of genetic eye diseases and their available family members were collected from our Pediatric and Genetic Clinic, Zhongshan Ophthalmic Center, Guangzhou, China. The clinical data were recorded and the genomic DNA was prepared in our laboratory at the State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center. Written informed consent in accordance with the tenets of the Declaration of Helsinki was obtained from patients' guardians prior to the collection of clinical data and venous blood samples. This study was approved by the institutional review board of the Zhongshan Ophthalmic Center. Genomic DNA was prepared from leucocytes obtained from venous blood using a previously described method (Wang et al. 2010).

Mutation detection

Our laboratory has so far collected in-house exome sequencing data from 2133 probands with different forms of HRD, including WES data obtained from 1139 probands, TES data obtained from 994 probands and Sanger sequencing from one proband. The procedure used to perform WES was described in our previous study (Jiang et al. 2015; Li et al. 2015a). In brief, exome as well as their adjacent intronic regions (at least 20 bp) were captured using the Agilent SureSelect Human All Exon Enrichment Kit (50M; Agilent, Santa Clara, CA, USA) array. The exome-enriched DNA fragments from the sample were sequenced by the Illumina HiSeq system (Illumina, San Diego, CA, USA) with an average sequencing depth of 125-fold. The reads were aligned with the consensus sequence (UCSC hg19) to detect variants by the Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/). Single nucleotide polymorphisms and small insertions and deletions (Indels) were detected by SAMTOOLS (http://samtools.sourceforge.net/) on the basis of a Bayesian statistical algorithm. Variant annotations were performed using SnpEff (http://snpeff.sourceforge.net/) and ANNOVAR (http://annovar.openbioinformatics.org/en/latest/), whereas the functional prediction of variants was predicted by dbNSFP (http://varianttools.sourceforge.net/Annotation/DbNSFP). Targeting exome sequencing (TES) was performed in our laboratory and targeted 126 genes (including *RPE65* and many related genes) frequently mutated in genetic eye diseases in the Chinese population. Sequencing variations in *RPE65* were retrieved from the 2133 probands.

Variants detected by WES and TES were initially filtered by multi-step bioinformatics analyses, as described in our previous study (Jiang et al. 2015; Li et al. 2015a). Candidate variants were also filtered by comparing our data with those available in existing databases, including the Human Genome Mutation Database (http://www.

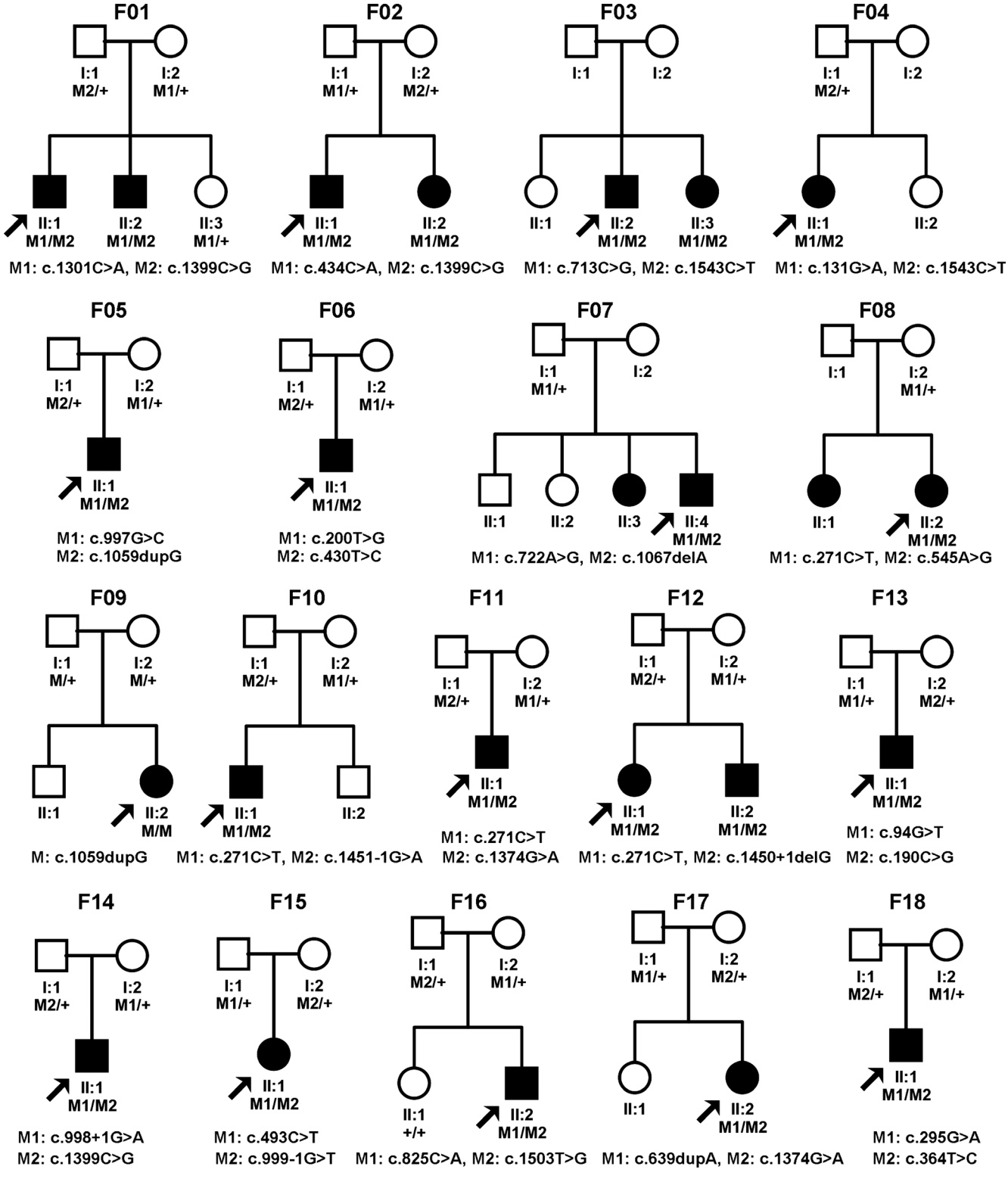

Fig. 1. Pedigrees of 18 families with biallelic *RPE65* mutations. Squares indicate male individuals while circles indicate females. Shading indicates an affected individual. Proband is indicated by arrows. Family numbers are on top of the pedigrees while mutations are listed under the pedigrees.

hgmd.cf.ac.uk/ac/index.php), the 1000 Genomes (http://phase1browser.1000genomes.org/) and the Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/). The possible impact of missense changes was predicted using the online tools SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) (Kumar et al. 2009) or PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) (Flanagan et al. 2010), MutationTaster (http://mutationtaster.org/) and CADD (https://cadd.gs.washington.edu/). Effect on splicing of intronic variants was predicted by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/ [in the public domain]) and Human

Table 2. Clinical information of the probands and affected siblings with biallelic RPE65 mutations.

Family ID	Clinic group	Mutation	Effect	Detection method	Gender	Age (year) at			Axial length or refraction	First symptom	Visual acuity		Fundus changes		ERG recording	
						Onset	1st exam	Last exam			Right	Left	Right	Left	Rods	Cones
F01-II:1	FAP	c.[1301C>A];[1399C>G]	p.[Ala434Glu];[Pro467Ala]	WES	M	4.7	5.7	7.9	21.49/ 21.44 mm at 5.7 years	NB	0.30	0.40	WD	WD	NA	NA
F01-II:2	FAP	c.[1301C>A];[1399C>G]	p.[Ala434Glu];[Pro467Ala]	SS	M	ECH	4.3	6.4	+2.75/+2.75 at 3.3 years	NB	0.40	0.40	WD	WD	Ext	SR
F02-II:1	FAP	c.[434C>A];[1399C>G]	p.[Ala145Asp];[Pro467Ala]	WES	M	3.3	3.3	NA	NA	NB	NA	NA	WD	WD	Ext	SR
F02-II:2	FAP	c.[434C>A];[1399C>G]	p.[Ala145Asp];[Pro467Ala]	SS	F	NA	8.0	NA	NA	NA	NA	NA	WD	WD	MiR	N
F03-II:2	FAP	c.[713C>G];[1543C>T]	p.[Ser238Cys];[Arg515Trp]	WES	M	ECH	26.0	NA	−3.25/−3.25 at 26 years	NB	1.00	1.00	WD	WD	MiR	MiR
F03-II:3	FAP	c.[713C>G];[1543C>T]	p.[Ser238Cys];[Arg515Trp]	SS	F	ECH	24.0	NA	−2.75/−1.75 at 24 years	NB	0.90	0.80	WD	WD	MoR	MiR
F04-II:1	FAP	c.[131G>A];[1543C>T]	p.[Arg44Gln];[Arg515Trp]	TES	F	2.0	10.0	NA	21.00/ 20.90 mm at 10 years	NB	0.40	0.40	WD, TD	WD, TD	SR	SR
F05-II:1	LCA*	c.[997G>C];[1059dupG]	p.[Gly333Arg];[Lys354Glufs*11]	SS	M	1.0	2.0	NA	NA	PV	NA	NA	TD, AV	TD, AV	Ext	Ext
F06-II:1	LCA†	c.[200T>G];[430T>C]	p.[Leu67Arg];[Tyr144His]	WES	M	ECH	2.1	8.8‡	+6.25/+5.00 at 2.1 years	NYS	0.10	0.10	TD, AV	TD, AV	Ext	Ext
F07-II:4	LCA	c.[722A>G];[1067delA]	p.[His241Arg];[Asn356Metfs*17]	WES	M	ECH	15.0	NA	−0.75/−1.75 at 15 years	NYS, PV	0.15	0.06	TD, MD	TD, MD	Ext	SR
F08-II:2	LCA	c.[271C>T];[545A>G]	p.[Arg91Trp];[His182Arg]	WES	F	ECH	19.0	NA	20.93/ 20.84 mm at 19 years	NYS, PV	0.03	0.04	TD, MD, AV	TD, MD, AV	Ext	Ext
F09-II:2	LCA	c.[1059dupG];[1059dupG]	p.[Lys354Glufs*11];[Lys354Glufs*11]	WES	F	0.3	0.3	NA	NA	PV	LP	LP	TD, AV	TD, AV	Ext	Ext
F10-II:1	LCA	c.[271C>T];[1451-1G>A]	p.[Arg91Trp];[splicing]	WES	M	0.3	4.9	NA	+5.00/+5.25 at 4.9 years	PV, NYS	0.10	0.15	TD, AV	TD, AV	NA	NA
F11-II:1	LCA	c.[271C>T];[1374G>A]	p.[Arg91Trp];[Trp458*]	WES	M	0.5	0.5	NA	NA	PV	LP	LP	TD, AV	TD, AV	Ext	Ext
F12-II:1	LCA	c.[271C>T];[1450+1delG]	p.[Arg91Trp];[splicing]	TES	F	ECH	4.7	NA	−3.25/−0.50 at 4.7 years	PV, NYS	NA	NA	TD, AV	TD, AV	Ext	Ext
F13-II:1	EORD	c.[94G>T];[190C>G]	p.[Gly32Cys];[Gln64Glu]	WES	M	3.0	5.3	NA	0/−0.25 at 5.3 years	PV	0.30	0.40	TD	TD	Ext	Ext
F14-II:1	EORD	c.[998+1G>A];[1399C>G]	[splicing];[p.(Pro467Ala]	TES	M	2.5	2.5	5.7‡	−5.25/−5.75 at 3.5 years	NB	0.20	0.20	TD	TD	Ext	Ext
F15-II:1	EORD	c.[493C>T];[999-1G>T]	p.[Gln165*];[splicing]	TES	F	2.3	3.2	NA	+2.50/+2.75 at 3.2 years	NB	FC	FC	TD, AV	TD, AV	Ext	Ext
F16-II:2	EORD	c.[825C>A];[1503T>G]	p.[Tyr275*];[Tyr501*]	TES	M	1.7	7.7	NA	−2.50/−1.25 at 7.7 years	PV, NB	0.15	0.30	TD, AV	TD, AV	Ext	Ext
F17-II:1	EORD	c.[639dupA];[1374G>A]	p.[Ala214Serfs*20];[Trp458*]	TES	F	2.0	5.2	NA	+3.00/+3.00 at 5.2 years	NYS	0.10	0.10	TD	TD	NA	NA
F18-II:1	HH	c.[295G>A];[364T>C]	p.[Val99Ile];[Tyr122His]	WES	M	2.4	2.4	6.0‡	+6.00/+6.50 at 2.4 years	Esotropia	1.00	0.50	Mild TD§	Mild TD§	N	N

AV = attenuated vessels, ECH = early childhood, EORD = early-onset retinal degeneration, Ext = Extinguished, F = female, FAP = fundus albipunctatus, FC = finger counting, HH = high hyperopia, LCA = Leber congenital amaurosis, LP = light perception, M = male, MiR = Mildly reduced, MoR = Moderately reduced, N = Normal, NA = Not available, NB = night blindness, NYS = nystagmus, PV = poor vision or no pursuit of objects, SR = severely reduced, SS = Sanger sequencing, TD = tapetoretinal degeneration, TES = targeting exome sequencing, WD = white dot deposits in mid-peripheral retina, WES = whole exome sequencing.

* RPE65 mutations in this family were detected by Sanger sequencing and reported in our previously study (Li et al. 2011).

† RPE65 mutations in this family were detected by WES and described in our previous study (Chen et al. 2013).

‡ Visual acuity for these children was obtained at last examination.

§ Normal appearance at posterior fundus but very mild tapetoretinal degeneration in mid-peripheral retina.

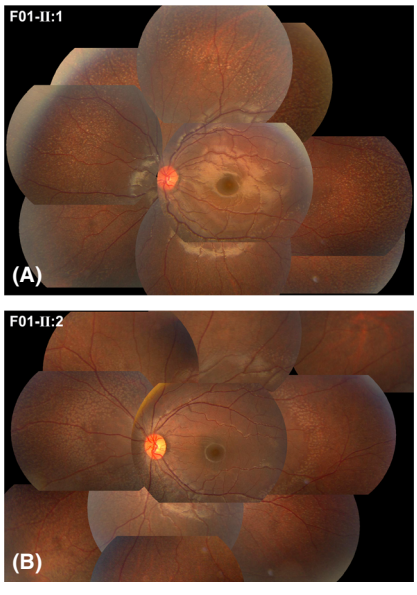

Fig. 2. Photos demonstrating the fundus changes typical of fundus albipunctatus-like changes in two affected brothers in family F01. A number of grey-white dots were present in the mid-peripheral retina. The family number and individual ID number that correspond to those shown in Fig. 1 and Table 2 are listed on the top left corner of each photo, as shown in Figs 3–5.

Splicing Finder (http://umd.be/HSF3/). Candidate disease-associated variants were confirmed by Sanger-dideoxy sequencing, and cosegregation in family members was further evaluated.

Probands with biallelic rare variants in *RPE65* were selected for further analysis. In rare cases, one heterozygous mutation, c.1430A>G (p.Asp477Gly) (Bowne et al. 2011), has been reported to cause autosomal dominant retinitis pigmentosa, and this mutation was therefore checked.

Phenotype characterization

Routine clinical data obtained from all probands with biallelic *RPE65* mutations were reviewed. Additional examinations were carried when necessary, including electroretinogram and fundus photographs. Parents or siblings were examined if available. Phenotypes were classified based on symptoms, visual acuity, fundus changes and electroretinogram data. Two previously reported families with LCA in which *RPE65* mutations were detected by either WES or Sanger sequencing were also included in the current analysis.

Results

Biallelic mutations detected in *RPE65*

In total, biallelic mutations were detected in 18 families (Table 1, Fig 1), with 17 detected by WES or TES and one was detected by Sanger sequencing. These mutations involved 27 different rare nucleotide changes, including missense variants (16), stopgain (four), frameshift insertions (two), frameshift deletion (one), splicing donors (two) and splicing acceptors (two). Twelve mutations were novel while the other 15 have been reported in previous studies (Table 1). Of these 27 variants, 26 were predicted to be damaging by routine SIFT or PolyPhen-2 tools. The rest one, p.Tyr122His was detected in a proband with high hyperopia and predicted to be benign or tolerated by Polyphen-2 and SIFT but to be possible disease causing by MutationTaster and CADD. All but one mutation (c.295G>A) was very rare,

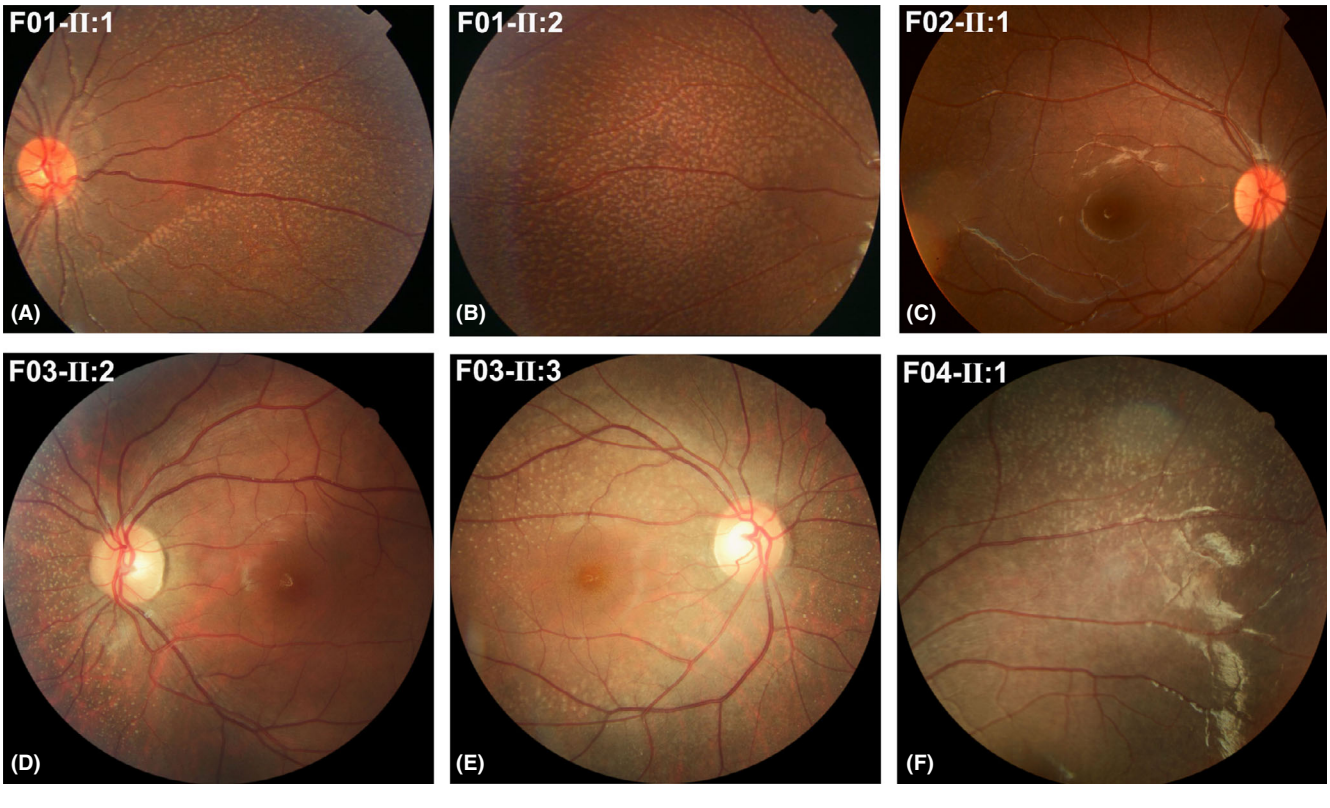

Fig. 3. Fundus photos showing fundus albipunctatus-like changes. A similar feature consisting of grey-white dots was observed among patients from different families and patients within the same family. These photos also show the varied numbers, varied sizes and varied densities of the grey-white dots that were observed among different families.

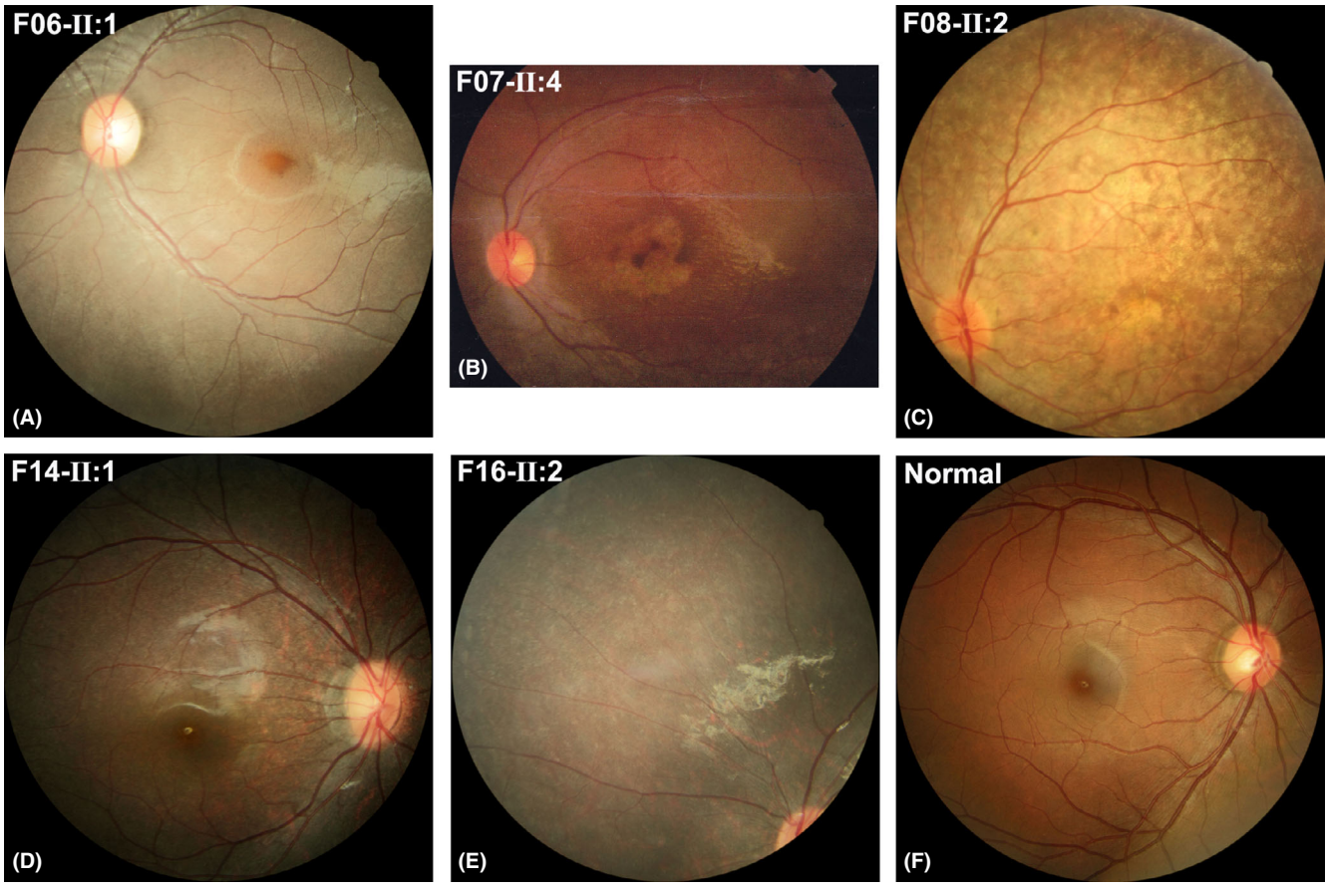

Fig. 4. Tapetoretinal degeneration, generalized or located mainly in the mid-peripheral region, mild or severe, was observed in different patients (A–E). A fundus photo of a normal individual that served as a control (F).

with no homozygote detected based on the existing database, and the frequencies ranged from none to 0.0002 for 27 mutations). The c.295G>A (p.Val99Ile) mutation had a frequency of 0.0012 for all and 0.0048 for Southern Han, with only one homozygote detected based on the ExAC and 1000 Genomes database. These mutations were confirmed by Sanger sequencing and cosegregated with the disease in the families (Fig 1 and Fig. S1). Of the 18 families with RPE65 mutations, parental analysis was available in 17 families and the results suggested that the two mutations in the 17 families were located in different chromosomes while the parents from one family (F03) were not available. It is difficult to cover the c.713C>G and c.1543C>T mutations in family F03 by the same primers because of a 9738 bp distance between them. However, these two variants are more likely to be biallelic as these two variants were neither observed in the 2132 of the 2133 HRD cases nor in 2871 cases with non-HRD diseases in our in-house exome data. None of the probands in the 18 families had mutations in the *RDH5* or *RLBP1* genes that are known to be associated with FA. The c.1430A>G (p.Asp477Gly) variant associated with autosomal dominant retinitis pigmentosa was not detected in any of the 2133 probands.

Phenotypic characterization

Of the 24 affected individuals in the 18 families, ophthalmological data were available for 21, including all 18 probands and three affected siblings (Table 2). The initial symptoms in the probands were night blindness in seven families, poor vision or no pursuit of objects in six families, nystagmus with poor vision in three families, nystagmus in one family and suspected esotropia in one family. Symptoms appeared as early as 4 months old and no later than 5 years old. Visual acuity ranged from finger counting to normal. Fundus changes ranged from very mild to easily recognizable. Two major types of fundus changes were visualized, including white dot deposits in the mid-peripheral retina (Figs 2 and 3) and tapetoretinal degeneration (Fig. 4). Macular degeneration was obvious in two probands who also had tapetoretinal degeneration (Fig. 4). Bone-spicule-like pigment deposits were not observed in any of the 21 affected individuals. Cone and rod responses on electroretinogram varied from nonrecordable to normal, although typical fundus changes were present. The diseases in the 18 families with *RPE65* biallelic mutations could be classified into four groups: LCA in eight families, EORD in five families, FA-like changes in four families and high hyperopia in one family.

New common and rare phenotypes

Of the four families with FA-like changes, three had affected siblings (Fig. 1, F01–F03), and one was a singleton case (Fig. 1, F04), suggesting an autosomal recessive trait. Similar fundus changes that were typical for FA-like changes were documented in the mid-peripheral region of the retina (Fig. 2 and 3). The type of fundus changes was almost identical between the eyes of each individual, between

affected siblings in three families (F01–F03) and similar among all seven patients from unrelated families (F01-II:1, F01-II:2, F02-II:1, F02-II:2, F03-II:2, F03-II:3 and F04-II:1). The initial symptoms observed in the seven patients with FA-like changes and *RPE65* mutations was night blindness, suggesting a milder phenotype than was found in those in whom poor vision was the initial sign. Five of the seven patients with FA-like changes had visual acuity better than 0.3 (F01-II:1, F01-II:2, F03-II:2, F03-II:3 and F04-II:1), and of these, two had visual acuity close to normal and exhibited mild or moderate changes on electroretinogram, even though they had typical retinal changes of FA-like changes (F02-II:2 and F03-II:2).

One proband in F18 initially visited our clinic due to suspected esotropia. A clinical examination revealed high hyperopia at 2 years and 5 months of age. The boy had a refraction of +6.00/OD and +6.50/OS at that time and a refraction of +5.25/OD and +6.25/OS when he reached 5 years and 2 months old. This patient had two variants (c.295G>A/p.Val99Ile and c.364T>C/p.Tyr122His) that were predicted to be benign by PolyPhen-2, damaging or tolerated by SIFT, and disease causing by MutationTaster and CADD. A recent examination showed a normal macula on optical coherence tomography, normal cone and rod responses on an electroretinogram performed at 6 years and 3 months of age, and mild tapetoretinal degenerative changes in the mid-peripheral retina (Fig. 5). High hyperopia or short axial length was recorded in six of the 21 affected individuals (Table 2). Long-term follow-up will be performed to document any progress in fundus retinal changes, as progress from a normal-like retina to typical tapetoretinal degeneration was observed in one proband (F05-II:1) with LCA and *RPE65* mutations after a follow-up of 6 years (Fig. 5).

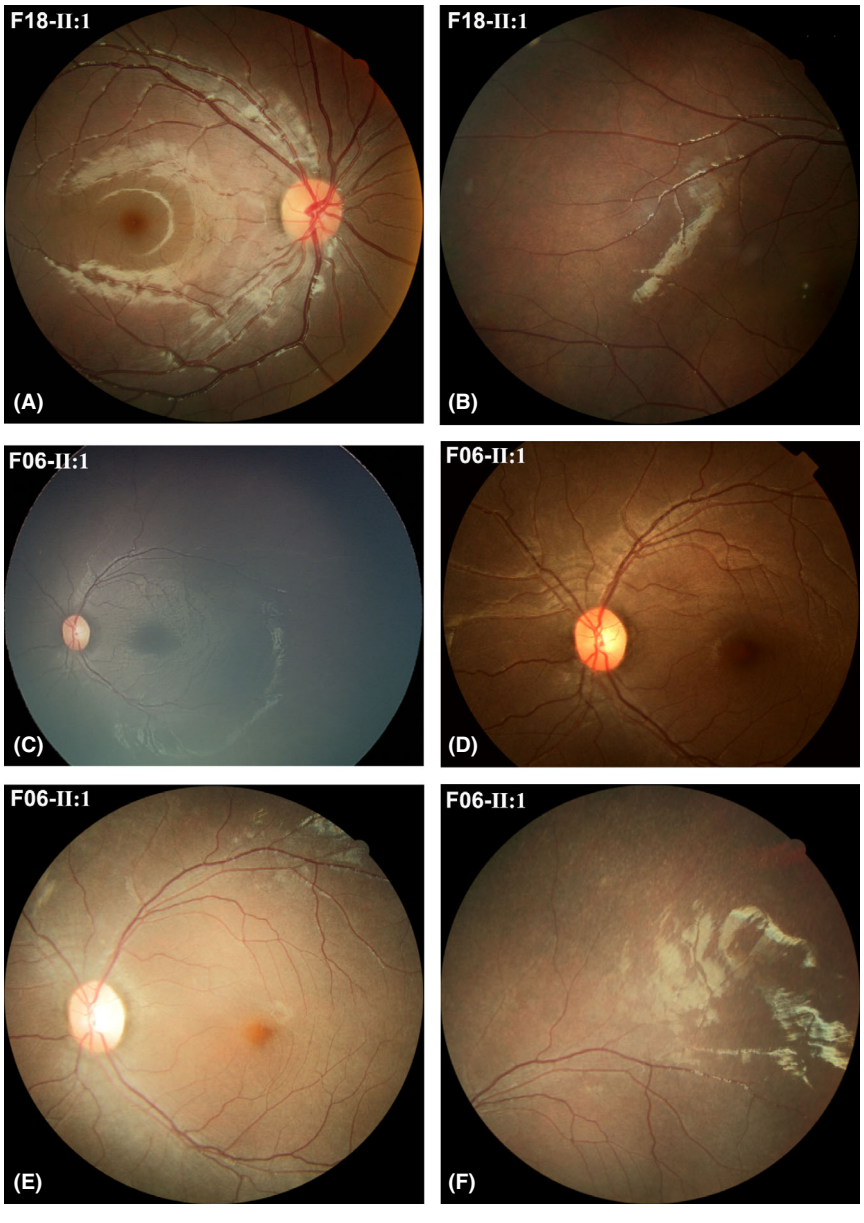

Fig. 5. Mild fundus change and progression in retinal degeneration. (A, B) Fundus photos obtained from F18-II:1, who had high hyperopia. The photos were taken when the patient was 6 years and 3 months old and showed a normal-like posterior fundus and mild degenerative changes at the mid-peripheral retina. He had a corrected visual acuity of 1.0 in the right eye and 0.5 in the left eye. (C–F) Fundus photos obtained from F06-II:1. The photos were taken when the patient was 2 years and 2 months old (C), 5 years old (D) and 8 years and 10 months old (E, F). The fundus changes were insignificant or very minor in the early stage (C, D) but an obvious tapetoretinal degeneration was observed in the posterior (E) and mid-peripheral retina (F) when the patient was 8 years and 10 months old.

Discussion

Of the 2133 families with HRD, 269 had an initial diagnosis of LCA. Therefore, biallelic *RPE65* mutations contributed to approximately 3.0% (8/269) of LCA and 0.8% (18/2133) of HRD cases. Clinically, diseases in 13 of the 18 families could be classified as LCA or EORD, which are common phenotypes previously reported to be associated with *RPE65* mutations (Gu et al. 1997; Marlhens et al. 1997; Morimura et al. 1998; Thompson et al. 2000; Simovich et al. 2001; Yzer et al. 2003; Booij et al. 2005; El Matri et al. 2006; Simonelli et al. 2007; Li et al. 2009; Xu et al. 2012; Kabir et al. 2013; Verma et al. 2013; Astuti et al. 2016; Katagiri et al. 2016). In the remaining five families with biallelic *RPE65* mutations, we unexpectedly found that the phenotypes were FA-like changes in four families and high hyperopia in one family.

Fundus albipunctatus-like (FA-like) changes were observed in seven patients from four families, suggesting that FA-like changes are a common phenotype of *RPE65*-associated retinopathy. Several lines of evidence support this notion, including the fact

that biallelic *RPE65* mutations were detected in all seven patients in four families, while mutations in *RDH5* and *RLBP1* were excluded, and similar fundus changes typical of FA-like changes were found in different families as well as in different affected siblings. Previously, FA-like changes had been described in only a few cases with *RPE65* mutations; these included an 18-year-old woman from Denmark with compound heterozygous mutations (IVS1+5G>A and c.344T>C) (Schatz et al. 2011), a 7-year-old British girl with c.433G>A and c.886dupA mutations (Hull et al. 2016), a 3-year-old Chinese boy with c.639_640insA and c.982C>T mutations (Yang et al. 2017) and two Japanese patients with c.[1543C>T];[683A>C] or c.[1028T>A];[683A>C] mutations (Katagiri et al. 2018). It remains unknown why FA-like changes is commonly observed in Chinese patients with biallelic *RPE65* mutations. As observed in the current study, patients with FA-like changes may have relatively good or even normal visual acuity as well as preserved rod-cone function for many years (Hull et al. 2016). The mutations identified in all four families are missense mutations, and two of the mutations (c.1399C>G and c.1543C>T) were shared by two different families. This may indicate that some missense mutations are associated with this phenotype. *RPE65* is not usually screened in patients with FA-like changes in traditional mutational analysis, especially in phenotype-guided candidate screening. Another possibility is that FA-like changes may be seen in only certain periods of the disease course, and longer-term follow-up may resolve this issue. Nevertheless, recognizing this new common phenotype may be helpful for identifying patients with *RPE65*-associated retinopathy so that they can benefit from potential *RPE65* gene therapies.

It is unusual that one proband had high hyperopia and biallelic *RPE65* mutations. These mutations may not necessary be causative, but they may have caused a mild phenotype: high hyperopia with mild or late-onset retinal dystrophy. Currently, no firm evidence demonstrates this causation, but several findings imply a disease-causing effect: (1) high hyperopia was a common feature as it was recorded in six of the 21 affected individuals examined in this study (Table 2); (2) *RPE65* biallelic mutations are very rare in both our in-house data and existing database; (3) of the two mutant alleles, one (c.364T>C) is novel and absent in the existing database, while the other was a known causative mutation; and (4) a mild phenotype consisting of normal visual acuity or late-onset retinal dystrophy was previously associated with hypomorphic mutant alleles (Lorenz et al. 2008; Li et al. 2015a,2015b; Hull et al. 2016; Samardzija et al. 2016). Similarly, progress from a normal-like retina to typical tapetoretinal degeneration was observed in one proband (F05-II:1) with LCA and *RPE65* mutations after 6 years of follow-up (Fig. 5). We expect that further studies using longer-term follow-up will determine whether there is any progress in the fundus retinal changes observed in the proband F18-II:1.

In summary, genotype-guided phenotype characterization not only leads to the identification of additional common phenotypes associated with biallelic *RPE65* mutations but also reveals the overall frequency of *RPE65*-associated eye diseases. Similarly, more information related to many other genes can be obtained by performing genotype-guided phenotype clarification than when using traditional phenotype-guided candidate gene analysis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Confirmation of mutations and validation in available family members in RPE65 by Sanger sequencing. Pedigrees are shown in the left column. Sequence chromatography of mutations in each family is shown in the middle and right columns with individual number on the left and mutations above sequences.