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Seven novel variants expand the spectrum of *RPE65*-related Leber congenital amaurosis in the Chinese population

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Purpose: To screen *RPE65* in 187 families with Leber congenital amaurosis (LCA).
Methods: Sanger sequencing and/or targeted exome sequencing was employed to identify mutations in the *RPE65* gene, and intrafamilial cosegregation analysis if DNA was available. In silico analyses and splicing assay were used to evaluate the variants' pathogenicity.
Results: Genetic analysis revealed 15 mutations in *RPE65* in 14 pedigrees, including one splice-site mutation, one frameshift mutation, three nonsense mutations, and ten missense mutations. Of the mutations identified in *RPE65*, seven are novel associated with LCA, including five missense variants (c.124C>T, c.149T>C, c.340A>C, c.425A>G, and c.1399C>G) and two indel (insertions or deletions) variants (c.858+1delG and c.1181_1182insT). In vitro splicing assay was performed to evaluate the functional impact on RNA splicing of novel mutations if two of three in silico analyses were predicated to be non-pathogenic at the protein level. Among these 15 variants, 14 were classified as 'pathogenic variants,' and a variant (c.124C>T) was 'variants with uncertain significance' according to the standards and guidelines of the American College of Medical Genetics and Genomics.
Conclusions: Mutations in *RPE65* were responsible for 11 of the cohort of 187 Chinese families with LCA, which expands the spectrum of *RPE65*-related LCA in the Chinese population and potentially facilitates its clinical implementation.

Leber congenital amaurosis (LCA) is an array of genetic childhood-onset retinal dystrophies (RDs), with an estimated prevalence of 1:50,000–1:100,000 [1]. Due to this condition, up to 5% of all with RD and about 20% of children attend schools for the blind [2]. More than 10% of affected individuals have mutations in the *RPE65* gene (locus name LCA2; OMIM #204100) encoding the protein with a molecular weight of 65 kDa specific in the RPE, which functions as the all-trans-retinyl esters to 11-cis-retinol isomerase and the lutein to mesozeaxanthin isomerase in the visual cycle [2-6]. Regeneration of visual pigment in rod photoreceptors after light exposure is completely reliant on the isomerization of RPE-derived RPE65, and *RPE65* deficiency can lead to severe dysfunction of rod photoreceptors and then result in severe impairment of night vision from birth [7,8]. The functions of cone photoreceptors responsible for daylight vision, high visual acuity, and color discrimination are relatively preserved in childhood as retinal cones can get an alternative source of 11-cis [9]. Nevertheless, the function of cone photoreceptors can be progressively affected due to the knock-on effect on the progressive degeneration of the outer retina brought about by local accumulation of toxic retinyl esters [10]. In addition, *RPE65* defect may affect the production of mesozeaxanthin which is abundant at the fovea center and is hypothesized to protect cones from oxidative stress and blue light damage [6]. The progressive dysfunction of cone photoreceptors results in severe impairment of cones-mediating daylight vision by early adulthood [10].

In humans, more than 100 mutations in *RPE65* have been identified associated with LCA, over all of the gene's 14 exons and their boundaries, up to half of which are missense mutations. Despite the broad spectrum of disease-causing mutations in *RPE65* in different populations, the spectrum of *RPE65* mutations reported is still narrow in the Chinese population. With a combination of different genotyping techniques and the published guidelines and standards of the American College of Medical Genetics (ACMG), this study showed that mutations in *RPE65* are the cause of LCA in 11 Chinese families in a cohort of 187 Chinese families with LCA and identified the underlying 15 mutations in *RPE65*, adding seven novel variants to the spectrum of

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disease-causing mutations in *RPE65*. These data facilitate genetic counseling and the selection of patients with LCA who are eligible for therapeutic retinoid supplementation or gene augmentation.

METHODS

Patients and phenotyping: The Institutional Review Board (IRB) of Tongji Eye Institute of Tongji University School of Medicine, (Shanghai, China) approved this study and we performed its whole procedure according to the tenets of the Declaration of Helsinki. All participating family members provided informed written consent that has been endorsed by the respective IRBs and is consistent with the tenets of the Declaration of Helsinki. In this study, a total of 187 unrelated Chinese probands enrolled from the 16 provinces of China had been diagnosed with LCA by retina specialists. Another cohort ascertained in this study as healthy control comprised 200 ethnically matched unrelated individuals. Detailed ocular examinations and routine physical examinations were performed to obtain clinical data about these participants in this study. An approximate 5 ml blood sample was voluntarily provided by all participants, and DNA extraction kits from TianGen Biotech Company, Beijing, China were used to isolate total genomic DNA.

Targeted exome sequencing: About 5 µg genomic DNA of the probands from six unrelated families with LCA (Family 20041, 20061, 20071, 20289, 20314, and 20357) was quantified with a Thermo Scientific NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The 194 genes included in the targeted exome sequencing (TES) panel are shown in Appendix 1. We prepared libraries based on the Illumina standard protocol and loaded them on the NextSeq 500 (Illumina Inc., San Diego, CA) platform, on which paired-end sequencing was conducted with reads of 100 bp, providing at least 100X for each sample as the average coverage depth. The SOAPaligner program was used to align the sequence reads to the reference sequence of human genome (version hg19). Single-nucleotide polymorphisms (SNPs) were first detected with the SOAPsnp program after PCR duplicates were filtered by the Picard software , and indels were determined using the Burrows-Wheeler Aligner (BWA) and Genome Analysis Toolkit (GATK) programs. The candidate SNPs and indels were annotated using the following databases: 1000 Genomes Project, dbSNP150, YH database, HapMap Project, Exome Variant Server, and Global Variome shared LOVD (Leiden Open Variation Database). RPE65 (version_RPE65:180323) candidate variants of TES were validated with Sanger sequencing, and cosegregation analysis was performed to see whether the mutation cosegregates with LCA in each family. The pathogenicity was predicted via in silico analysis.

PCR and Sanger sequencing: The DNA from 187 LCA probands and 200 unrelated Chinese control individuals was screened for *RPE65* mutations. PCR amplified all 14 coding exons and flanking intronic sequences of *RPE65* (GenBank: NM_000329.2), purified and analyzed via Sanger sequencing. The PCR conditions and primers are listed in Appendix 2. The sequencing results were analyzed using Variation Surveyor (version 5.0.0) with the reference sequences from the NCBI database. When DNA from other family members of the probands was available, sequence analysis of the mutated fragment was performed for segregation analysis.

In silico analysis: Three in silico methods—Sorting Intolerant from Tolerant (SIFT), Polymorphism Phenotyping v2 (PolyPhen-2), and (Protein Variation Effect Analyzer (PROVEAN)—were used to evaluate the pathogenicity of the variants at the protein level. The allele frequency data for the identified variants were also assessed based on an available 'health control' exome data set from NHLBI Exome Sequencing Project Exome Variant Server, the Exome Aggregation Consortium (*ExAC*) database, and LOVD. Human Splicing Finder 3.0 (HSF 3.0) was used to predict the splicing defects caused by splicing variants and exonic variants predicted to be non-pathogenic in two of the three in silico methods at the protein level.

In vitro splicing assay: In vitro splicing assay was performed based on the comparative assay of the splice pattern of the *RPE65* fragment of wild-type (wt) and variant (var) constructed in minigene plasmid pCAS2 (a kind gift from Prof. A. Martins, University of Rouen, France) to evaluate the impact on splicing of the mutations that were predicted to have uncertain pathogenicity at the protein level [11,12]. For each mutation, the wild-type exons were PCR-amplified from human genomic DNA together with about 150 bp of flanking sequences. Amplification condition is to use a touchdown program (beginning at 64 ℃ -57 ℃, decreasing by 0.5 ℃ each cycle) with the primers (in Table S2). The fragments were inserted into the MluI and BamHI cutting sites of pCAS2. Minigenes carrying a mutation in *RPE65* were prepared with site-directed mutagenesis with the overlap PCR method and the construct pCAS2-WT *RPE65* as the template. The inserts of the constructs were sequenced to verify the accuracy of the constructs. The procedure for splicing minigene reporter assay, including HeLa cell transfection, Reverse transcriptase (RT)–PCR, and Sanger sequencing, was performed according to the description in Soukarieh et al. [13]. RT-PCR was performed (30 cycles of amplification) in a 25 µl reaction volume with OneStep RT-PCR kit (Qiagen,

Hilden, Germany), plus 100 ng total RNA, and forward and reverse primers (Fw: 5'-TGA CGT CGC CGC CCA TCA C-3', Rv: 5'-ATT GGT TGT TGA GTT GGT TGT C-3') and then RT-PCR product was performed by Sanger sequencing with above two primers . The protocol was followed according to the description in Soukarieh etal [13]. Three independent experiments were performed.

RESULTS

Among 14 *RPE65*-related families with LCA, the probands of six families (Family 20071, 20061, 20314, 20357, 20177, and 20425) have consanguineous parents (Figure 1 and Figure 2). Biallelic mutations in *RPE65* were identified in 12 families with LCA, and there are another two families (Family 20146 and Family 20388) with one allelic mutation in *RPE65* and an unknown mutation on the second chromosome, which are summarized in Table 1. Altogether, we identified 15 mutations in the *RPE65* gene, including ten missense mutations, a frameshift mutation, a splice-site mutation, and three nonsense mutations. Among them, eight mutations have been previously reported, and seven mutations are novel associated with LCA, including c.124C>T (p.L42F), c.149T>C (p.F50S), c.340A>C (p.N114H), c.425A>G (p.D142G), c.858+1delG (p.?), c.1181_1182insT (p.L395SfsX4), and c.1399C>G (p.P467A; Figure 1 and Figure 2). None of the mutations in *RPE65*, except c.272G>A, c.124C>T, and c.130C>T (reported less than four), were found in the ExAC Browser. In addition, all mutations in the study were not seen in the Exome Variant Server. In eight Chinese LCA pedigrees (Family 20071, 20041, 20061, 20357, 20077, 20361, 20177, and Family 20146) with other family members' DNA available, segregation analyses showed that those mutations cosegregated with the LCA phenotype.

In 14 families with mutations in *RPE65*, six families were evaluated with TES combined with Sanger sequencing, and eight families were evaluated with Sanger sequencing. The proband of Family 20289 who underwent TES carries compound heterozygous mutations in *RPE65* of a reported variant c.130C>T and a novel variant c.124C>T, as well as a reported heterozygous nonsense mutation c.6351G>A (p.Trp2117X) in *GPR179,* (Gene ID: 440435, OMIM 614565) which is a retinal disease–causing gene of related to complete congenital stationary night blindness with the model of autosomal recessive inheritance.

Of the 15 mutations, the splice-site mutation c.858+1delG was novel, and 11 other mutations were predicted to be pathogenic at the protein level in two or all three of the in silico prediction programs. However, three (c.124C>T, c.272G>A, and c.1338G>T) were predicted to be non-pathogenic at the protein level with all or two of the three in silico methods. The variant c.124C>T affects highly conserved residue Leu[42] of human RPE65, but it was predicted to be non-pathogenic with SIFT and PROVEAN, and it does not change RNA splicing compared with the wild-type (data not shown) via minigene assay although this mutation is located in the middle of exon 3, which suggests future research is still warranted to confirm its pathogenicity. c.272G>A was previously reported to be associated with LCA and supported to be pathogenic by functional assessment that the isomerase activity RPE65 with p.Arg91Trp substitution due to this variant was less than 6% of wild-type *RPE65* [14]. The variant c.1338G>T without evidence to support that it is pathogenic at the protein level was located first 3 exonic bp of exon 12. Therefore, in vitro in splicing minigene reporter assays were performed to learn whether the variants c.858+1delG and c.1338G>T have a functional impact on RNA splicing. Every variant is a single nucleotide substitution, and the bands were sequenced to confirm their composition (Figure 3). Minigene splicing demonstrated that the splice-site variant c.858+1delG may result in the same impact as c.859delG (p.Val287PhefsX38; Figure 3A) and the nonsynonymous variant c.1338G>T resulted in a corresponding loss of exon 12, which generated a premature stop codon and may lead to a complete loss of RPE65 (Figure 3B).

For the *RPE65*-related patients with LCA in the cohort, the age at onset was the first few months after birth, and their best corrected visual acuity (BCVA) was less than 0.2 over 30 years old except Family 20041_II:1 (Appendix 3). The patients in Family 20061 with the homozygous nonsense mutation (p.Q165X) in *RPE65* typically had ophthalmological symptoms at birth, which were characterized by roving eye movements and the inability to follow light or objects. In this family, the affected siblings phenotyped in their second decade had disc pallor with attenuated vessels and salt and pepper fundus with peripheral RPE mottling (Figure 4A,B). The younger sibling had a few early alterations in the macula (Figure 4A), and the elder sibling also revealed macular scarring (Figure 4B). The patient in Family 20041 carrying two *RPE65* variants (p.R91Q and p.L395Sfs*4) presented with symptoms of nystagmus, lack of fixation, and night blindness and/or orientation difficulties within the first year of age. This patient had severely constricted visual fields at the age of 15 years. The fundi were generally characterized by retinal degeneration and attenuated vessels with later signs of optic nerve atrophy and in this patient, mottled or bone spicule-like pigmentations (Figure 4C). Several previous studies described a similar phenotype in individuals with LCA with mutations in *RPE65*. Optical coherence tomography showed

reduced retinal thickness in these three patients with LCA (Figure 4D–F).

DISCUSSION

LCA is a rare inherited retinal degeneration, and thus far, more than 21 LCA-causing genes have been identified. As one of these mutated genes, *RPE65* is expressed in high concentrations in the RPE, and RPE65 functions as the retinoid isomerase enzyme indispensable for the production of chromophore to form the visual pigment in retinal photoreceptors [4,5,7,8]. Congenital reduction or even a lack of chromophore and progressive photoreceptor degeneration due to *RPE65* deficiency result in progressive and severe impairment of vision [8]. Due to significant effects shown in clinical trials, gene therapy for *RPE65*-related LCA with adenoassociated virus (AAV) recombinant vectors has received approval for clinical implementation [3,15,16]. In this study, we identified 15 potentially pathogenic mutations in 14 of 187 unrelated Chinese families with LCA, and mutations in *RPE65* are the cause of 11 Chinese families with LCA. These unrelated patients from 14 families were found to have mutations in *RPE65*, indicating the possibility of treatment with *RPE65* gene therapy.

The spectra distribution of mutations in *RPE65* in different populations is remarkably different. Cases of *RPE65*-related LCA have been reported more often in European and American families, and rarely reported in Chinese families. The mutations in the reports from Chinese populations include 14 missense mutations and seven presumptive null mutations (three nonsense, one splice-site, and three frameshift mutations) [17-26]. Of 14 *RPE65*-associated families previously reported in the Chinese population,

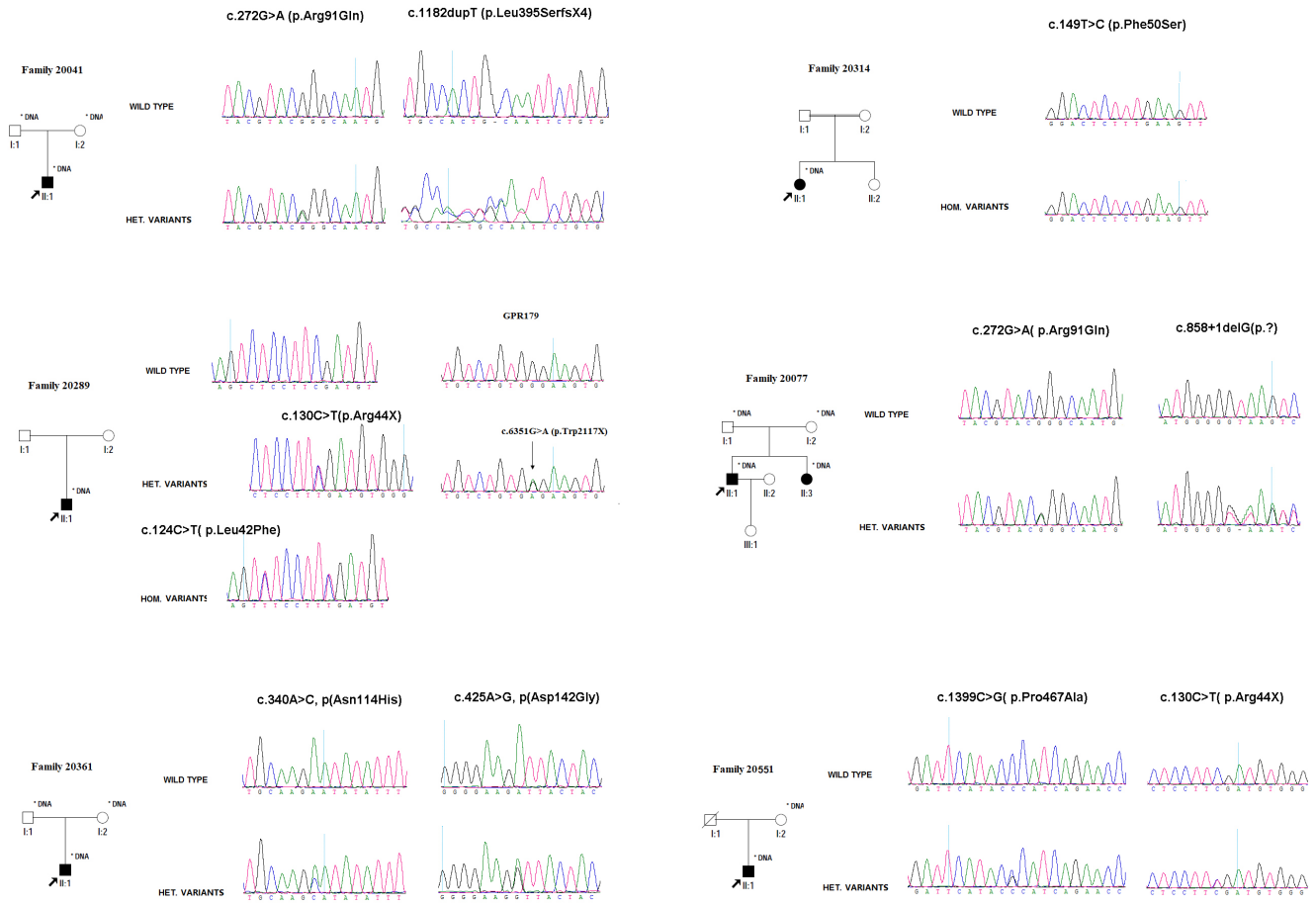

Figure 1. Pedigrees and sequence chromatograms of six families with novel mutations in *RPE65*. Pedigrees and sequence chromatograms of *RPE65*-related families. Pedigrees of six families show their potential inheritance model. Empty symbols: healthy controls, filled symbols: affected patients. Individuals genotyped with available DNA are marked with asterisks. Probands are pointed out by arrows, squares indicate males, and circles indicate females. A reported heterozygous nonsense mutation, c.6351G>A (p.Trp2117X), in *GPR179* was identified in Family 20289.

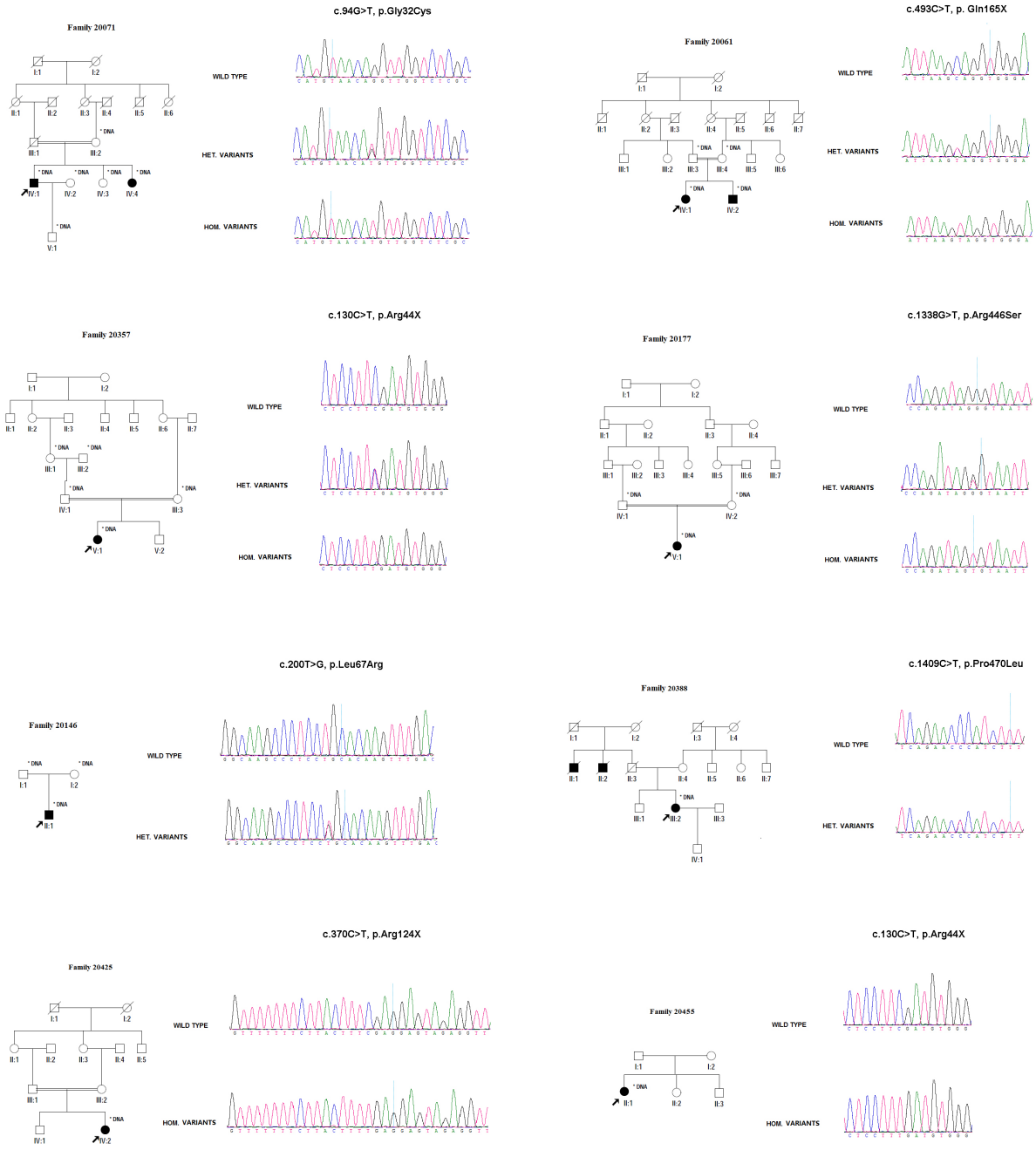

Figure 2. Pedigrees and sequence chromatograms of eight families with reported mutations in *RPE65*. Pedigrees and sequence chromatograms of *RPE65*-related families. Pedigrees of six families show their potential inheritance model. Empty symbols: healthy controls, filled symbols: affected patients. Individuals genotyped with available DNA are marked with asterisks. Probands are pointed out by arrows, squares indicate males, and circles indicate females.

only homozygous mutations were identified by Ruifang Sui's group and Juan Bu's group, and the other families all have compound heterozygous mutations in *RPE65* [18-26]. Based on the previous reports about mutations in *RPE65*, the variant c.200T>G (p.L67R) may be a founder mutation in the Chinese population (the heterozygous variant in five separate families), while the variant c.130C>T (p.R44X) is likely to be a founder mutation (the heterozygous variant in two unrelated families and the homozygous variant in another two unrelated families) according to this study. The present study expands

Table 1. Fifteen different RPE65 variants identified in the probands from unrelated 14 families.

Family ID	Exon	Variation nucleotide	Protein	Status	Type	Pathogenicity Prediction in protein level			ExAC	Methods	reported in literatures and RPE65 LOVD database
						PolyPhen-2	SIFT	PROVEAN			
20041	4	c.272G>A	p.Arg91Gln	Het.	Missense	Benign (0.283)	Tolerant −0.59	Neutral (−0.40)	4/120746	TES+Sanger	Y
	11	c.1182dupT	p.Leu395SerfsX4	Het.	Frameshift	-	-	-	NONE		N
20061	5	c.493C>T	p. Gln165X	Hom.	Nonsense	-	-	-	NONE	TES+Sanger	Y
20071	2	c.94G>T	p.Gly32Cys	Hom.	Missense	Probably damaging (0.985)	Damaging (0.00)	Deleterious (−8.07)	1/118428	TES+Sanger	Y
20077	4	c.272G>A	p.Arg91Gln	Het.	Missense	Benign (0.283)	Tolerant (0.59)	Neutral (−0.40)	4/120746	Sanger	Y
	8	c.858+1delG	p.?	Het.	Splicing variation	-	-	-	NONE		N
20146	3	c.200T>G	p.Leu67Arg	Het.	Missense	Probably damaging (0.998)	Damaging (0.00)	Deleterious (−4.64)	NONE	Sanger	Y
/		unknown variant on 2nd chromosome	/	/	/	/	/	/	/	/	/
20177	12	c.1338G>T	p.Arg446Ser	Hom.	Missense	Benign (0.001)	Tolerant (0.76)	Neutral (−1.09)	NONE	Sanger	Y
20289 †	3	c.124C>T	p.Leu42Phe	Het.	Missense	Probably damaging (0.997)	Tolerant (0.2)	Neutral (−2.05)	1/121396	TES+Sanger	N
	3	c.130C>T	p.Arg44X	Het.	Nonsense	-	-	-	4/121406		Y
20314	3	c.149T>C	p.Phe50Ser	Hom.	Missense	Probably damaging (1.000)	Damaging (0.01)	Deleterious (−6.28)	NONE	TES+Sanger	N
20357	3	c.130C>T	p.Arg44X	Hom.	Nonsense	-	-	-	4/121406	TES+Sanger	Y
20361	4	c.340A>C	p.Asn114His	Het.	Missense	Probably damaging (0.998)	Damaging (0.00)	Deleterious (−3.97)	NONE	Sanger	N
	5	c.425A>G	p.Asp142Gly	Het.	Missense	Benign(0.205)	Damaging (0.01)	Deleterious (−5.95)	NONE		N
20388	13	c.1409C>T	p.Pro470Leu	Het.	Missense	Probably damaging (0.998)	Damaging (0.00)	Deleterious (−8.72)	NONE	Sanger	Y
/		unknown variant on 2nd chromosome	/	/	/	/	/	/	/	/	/
20,425	5	c.370C>T	p.Arg124X	Hom.	Nonsense	-	-	-	5/120874	Sanger	Y

Family ID	Exon	Variation nucleotide	Protein	Status	Type	Pathogenicity Prediction in protein level			ExAC	Methods	reported in literatures and RPE65 LOVD database
						PolyPhen-2	SIFT	PROVEAN			
20,455	3	c.130C>T	p.Arg44X	Hom.	Nonsense	-	-	-	4/121406	Sanger	Y
20,511	13	c.1399C>G	p.Pro467Ala	Het.	Missense	possibly damaging(0.918)	Damaging (0.02)	Deleterious (−6.32)	NONE	Sanger	N
	3	c.130C>T	p.Arg44X	Het.	Nonsense	-	-	-	4/121406	Sanger	Y

Note: The proband of Family 20,289 marked with † sign carries the variation C.6351G>A (p.Trp2117X) in GPR179 gene. The items without data available are marked with backslash. Variants marked with hyphen are not necessary to be predicted or improper to be predicted their pathogenicity in protein level via SIFT, Polyphen-2 and PROVEN. Grayish lattices were splicing mutations and non-pathogenic results in protein level with all or two of three in silico approaches. Score ranges from 1 (Probably damaging), to 0.5 (Probably damaging) and to 0 (Benign) with cut-off score set at 0.05 in PolyPhen-2; Negative and positive scores indicate deleterious and neutral, respectively, with cut-off score set at −1 in PROVEAN; and score ranges from 0 (deleterious) to 1 (Tolerant) with cut-off score set at 0.05 in SIFT.

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the spectrum of LCA-related mutations with seven novel mutations and eight previously reported mutations added to the existing spectrum of the *RPE65* gene. Two individuals with LCA (Family 20135 and Family 20388) were identified with a heterozygous mutation only after sequence analysis of all coding regions of RPE65. The following possibilities could be ascribed to the failure to identify another allelic variant: 1) A duplication or deletion in another allele may not be detected as copy number variants analysis was not performed. 2) Deep-intronic regions were not covered in the screening, and thus, mutations in this part of the genome were not detected. 3) Variants in regulatory elements located far from *RPE65* cannot be ruled out. Moreover, the proband of Family 20289 carries a novel variant c.124C>T with unclear pathogenicity, another reported *RPE65* variant c.130C>T (p.R44X), and a reported heterozygous nonsense mutation c.6351G>A (p.W2117X) in *GPR179*, which suggest the possibility that c.124C>T combined with c.130C>T may not be enough or exclusive to cause LCA, and the mutation in *GPR179* may act as a genetic modifier in the LCA process of the proband of Family 20289 [27].

The *RPE65*-related patients with LCA in the cohort presented severe clinical phenotypes, such as early onset of blindness and mild improvement during puberty, which have been reported in previous studies [4,5,10,28]. In Family 20061 (homozygous c.493C>T, p.Q165X), the BCVA of patient IV:1 was hand movement (HM) before 21 years according to her medical history, and that of the patient IV:2 was 0.05/0.05, which shows variable expressivity, with environmental or other genetic factors involved (Table S3). However, there was no obvious difference observed in the visual impairment course between the two sibling patients in Family 20071 (homozygous c.94G>T, p.G32C; Table S3). The same *RPE65* variant c.272G>A (p.R91Q) is associated with LCA of Family 20077 and Family 20041, and slower progression of visual acuity of patient II:2 in Family 20041 than that of patient II:1 in Family 20077 might contribute to the different impacts between c.1182dupT (p.L395Sfs*4) and c.858+1delG (Table S3). Patient II:1 in Family 20146 with c.200T>G (p.L67R) and an unknown second allele has an no pursuit of light (NPL) of visual acuity at 27 years old while the patient with c.200T>G (p.L67R) and c.434C>A (p.A145D) reported in 2013 by Fu et al. [20] still had 0.2/0.2 of visual acuity at 41 years old, which suggested that the rapid progression of visual acuity might be attributed to an unknown second allele. Patient III:2 in Family 20388 still had finger counting (FC) of visual

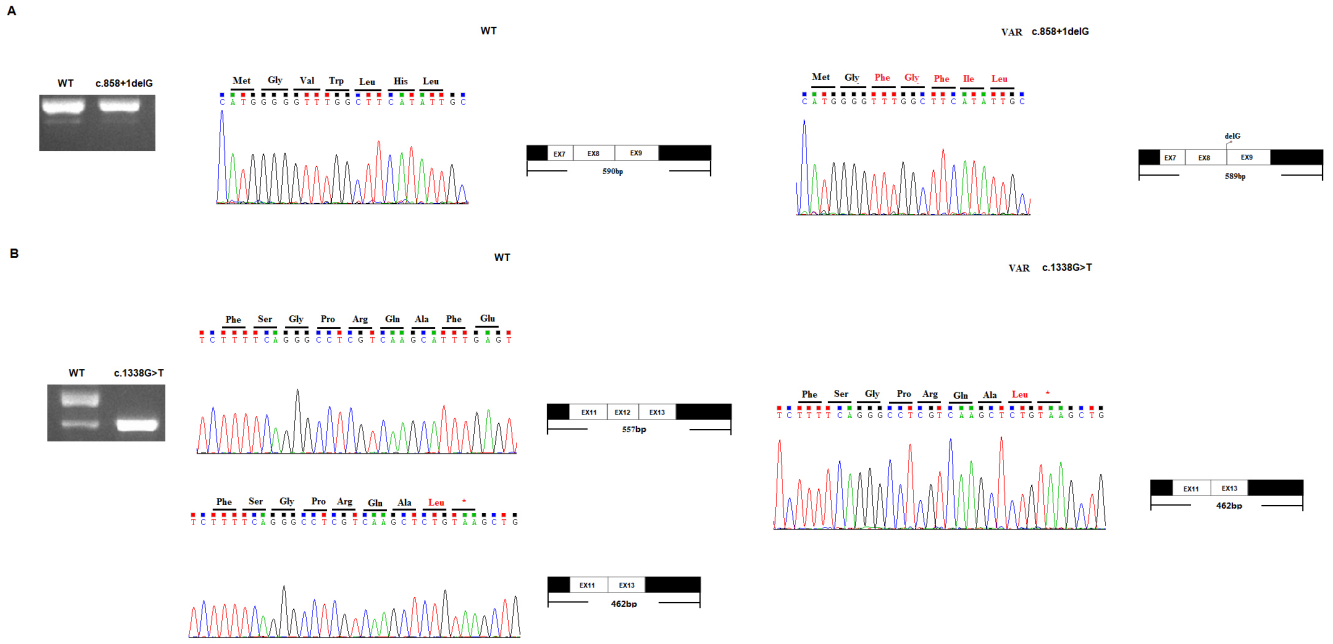

Figure 3. Splicing assay shows mutation-induced change in *RPE65* splicing. Gel electrophoresis of reverse transcriptase (RT)–PCR products for all tested constructs. The control is unmodified pCAS2 (a generous gift from Dr. A. Martins, University of Rouen, France). The differences between the respective wild-type (wt) and variation (var) band composition demonstrate the mutation-induced aberrant at the mRNA level. Dark exons: the first and last exon of the pCAS2 reporter minigene, white exons: the exons cloned in for *RPE65* mutation testing. We sequenced the band to verify the sequence of the splicing products with Sanger sequencing. **A**: Minigene-splicing assay of the c.858+1delG variant in *RPE65*. **B**: Minigene-splicing assay of the variant c.1338G>T in *RPE65*.

acuity at 41 years old with a variant c.1409C>T (p.P470L) and an unknown second allele that might be associated with her slower progression of visual acuity. Additionally, to our knowledge, missense mutations can result in decreased expression or change in catalytic activity of *RPE65*, or bring about a toxic effect. Ideally, a function study is helpful to clarify the mechanism of missense mutations associated with LCA while nonsense, frameshift, and splice-site mutations lead to presumptive null mutations in *RPE65* [14,29]. We assumed that the diverse impacts that such two kinds of mutations brought about might lead to the difference in phenotype. In this study, no obvious difference in phenotype was observed between patients with missense mutations and presumptive null mutations.

In summary, this study identified 15 LCA-related mutations in *RPE65* in a Chinese population, and 11 families in the study had LCA-causing mutations in *RPE65*. Therefore, mutations in *RPE65* were responsible for 5.88% (11/187) of the Chinese families with LCA in the cohort. Considering the developing retinal gene therapy for LCA, this work is helpful for further understanding the genetic etiology of LCA, and may potentially facilitate clinical implementation for families with LCA. Likewise, the review of all available aspects to date about mutations in *RPE65* in the Chinese population may also be of interest for further clinical management.

APPENDIX 1. TABLE S1. LIST OF 194 GENES INCLUDED IN THE PANEL USED IN THIS STUDY.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. TABLE S2. PRIMERS FOR SCREENING RPE65 VARIANTS.

To access the data, click or select the words "Appendix 2."

APPENDIX 3. TABLES3. CLINICAL CHARACTERISTICS OF RPE65-RELATED LCA PATIENTS IN THIS STUDY.

NOTE: F, female; M: male; FMB, first few months after birth; OD, oculus dexter; OS, oculus sinister BCVA, best corrected visual acuity; FC, finger counting; HM, hand movement; NPL, no pursuit of light; PV, poor vision; NYS,

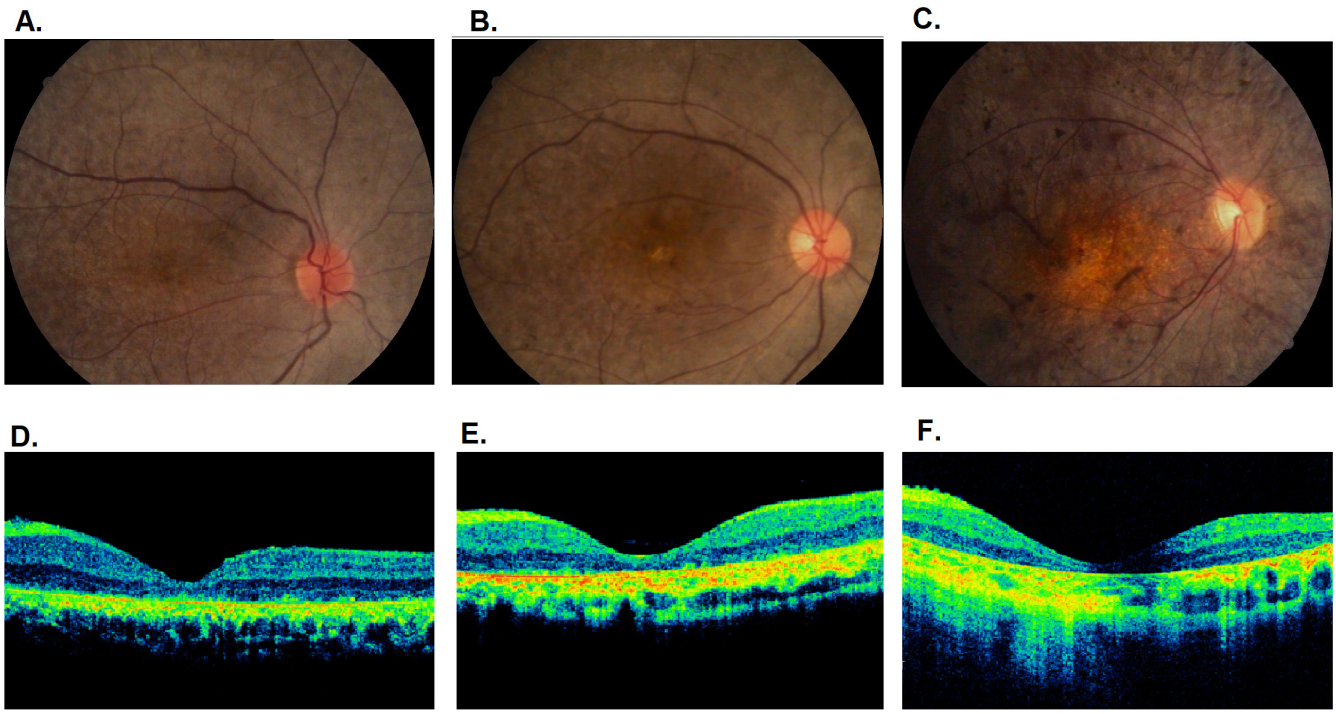

Figure 4. Fundus photograph and optical coherence tomography pictures of three patients with mutations in *RPE65*. **A**: Patient IV:1, a 23-year-old man in Family 20061, shows disc pallor, attenuated vessels with a few early alterations in the macula, and pepper fundus with peripheral RPE mottling. **B**: Patient IV:2 of Family 20061, a 32-year-old woman with a c.493C>T (p. Gln165X) mutation in *RPE65* (Family 20061), shows disc pallor, attenuated vessels, scar macula, with salt and pepper fundus. **C**: Patient II:2 of Family 20041, a 33-year-old man with two mutations in *RPE65* (p.Arg91Gln and p.Leu395SerfsX4), shows bone spicule-like formation in the fundus, attenuation of the retinal arterioles, and optic disc pallor. **D**–**F**: Optical coherence tomography demonstrates reduced retinal thickness in these three patients with LCA.

nystagmus; PM, pronounced maculopathy; PH, peripheral hyperpigmentation; RVA, retinal vascular attenuation. To access the data, click or select the words "Appendix 3."

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