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Comprehensive Molecular Diagnosis of a Large Chinese Leber Congenital Amaurosis Cohort

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Purpose. Leber congenital amaurosis (LCA) is an inherited retinal disease that causes early-onset severe visual impairment. To evaluate the mutation spectrum in the Chinese population, we performed a mutation screen in 145 Chinese LCA families.

Methods. First, we performed direct Sanger sequencing of 7 LCA disease genes in 81 LCA families. Next, we developed a capture panel that enriches the entire coding exons and splicing sites of 163 known retinal disease genes and other candidate retinal disease genes. The capture panel allowed us to quickly identify disease-causing mutations in a large number of genes at a relatively low cost. Thus, this method was applied to the 53 LCA families that were unsolved by direct Sanger sequencing of 7 LCA disease genes and an additional 64 LCA families. Systematic next-generation sequencing (NGS) data analysis, Sanger sequencing validation, and segregation analysis were used to identify pathogenic mutations.

Results. Homozygous or compound heterozygous mutations were identified in 107 families, heterozygous autosomal dominant mutations were identified in 3 families and an X-linked mutation was found in 1 family, for a combined solving rate of 76.6%. In total, 136 novel pathogenic mutations were found in this study. In combination with two previous studies carried out in Chinese LCA patients, we concluded that the mutation spectrum in the Chinese population is distinct compared to that in the European population. After revisiting, we also refined the clinical diagnosis of 10 families based on their molecular diagnosis.

Conclusions. Our results highlight the importance of a molecular diagnosis as an integral part of the clinical diagnotic process.

Keywords: Leber congenital amaurosis, next-generation sequencing, molecular diagnosis, mutation spectrum, Chinese LCA cohort

Leber congenital amaurosis (LCA; MIM 204000), a severe form of inherited retinal dystrophy, affects 1 in approximately 30,000 to 80,000 people in the general population, and accounts for more than 5% of all retinopathies and 20% of blindness in school-age children.[1–4] It is an early-onset retinal degenerative disease and its clinical features usually appear within the first year of life. The disease is characterized by nystagmus, fundus changes, severe congenital vision loss, and minimal or nondetectable electroretinogram (ERG).[5] Most cases of LCA follow an autosomal recessive pattern of inheritance, although some families with autosomal dominant LCA have been observed.[6]

The condition of LCA is highly heterogeneous with at least 22 genes, which are involved in a wide range of functional pathways, found to associate with the disease (available in the public domain at https://sph.uth.edu/Retnet/). Together, these genes account for approximately 75% of all LCA cases.[6–10] The large number of genes associated with LCA indicates that the mechanisms underlying this devastating disease are complex; as a result, an accurate molecular diagnosis is essential for an accurate clinical diagnosis and the proper treatment of LCA patients.

The heterogeneity of LCA is complicated further by the fact that LCA also can be caused by mutations in disease-causing genes that currently are associated with other types of retinopathies. For example, mutations in some syndromic and nonsyndromic retinal disease genes, such as *ALMS1*, *BBS4*, *CNGA3*, *IQCB1*, *KCNJ13*, and *MYO7A*, are known to associate with LCA or "LCA-like" phenotypes.[6,9,11–14] These phenomena are likely due to a combination of allelic differences, genetic background, and environmental modifications; they also indicate that many retinal diseases may, in fact, have similar disease mechanisms. On the other hand, at least seven LCA disease genes have been linked to juvenile retinitis pigmentosa (RP; MIM 268000): *CRX*, *CRB1*, *RPE65*, *RDH12*, *LRAT*, *MERTK*, *SPATA7*, and *TULP1*.[6,15] Similar associations have been made between known LCA disease genes and the clinical features of other retinal dystrophies, such as cone-rod dystrophy (*CRX*[16] and *AIPL1*[17]) and systemic diseases, such as Joubert and Meckel

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syndromes (*CEP290*).[18,19] As a result, interpretation of sequencing results often can be challenging.

Most LCA studies have been performed in Western populations, so the mutation frequency of each LCA disease gene in Europeans has been determined. In contrast, few studies have been done in East Asian populations and the mutation spectrum in this population remains unclear. To date, there are only two relatively small cohort studies involving Chinese LCA patients. Li et al.[20] screened 15 known LCA disease genes in 87 Chinese probands and identified 35 (66%) novel pathogenic mutations. The solving rate in this cohort was 50.6%, which is relatively low compared to that in European populations. Recently, Chen et al.[21] performed whole exome sequencing (WES) in 41 Chinese LCA patients and reported 17 novel variants (77%) after analyzing SNPs found in 19 known LCA disease genes. The solving rate revealed in this study is also approximately 50%. To reveal the mutation spectrum in the Chinese population and to accurately estimate the solving rate, we performed a comprehensive mutation analysis in 145 Chinese LCA families, which is the largest Chinese LCA cohort so far. Using targeted capture next-generation sequencing (NGS) or direct Sanger sequencing, we screened the coding region of 163 known retinal disease genes and identified 136 novel pathogenic mutations accounting for 81.4% of all mutations detected in this study. We concluded that the mutation spectrum in the Chinese population is distinct compared to that in the European population. Furthermore, we increased the solving rate to approximately 76.6% by analyzing mutations in other known retinal disease genes.

Materials and Methods

Recruitment of Subjects

All participants were identified at the Ophthalmic Genetics Clinic at Peking Union Medical College Hospital (PUMCH), Beijing, China. We defined LCA as severe visual impairment within the first year after birth, nystagmus, oculodigital sign, and severely reduced or nondetectable ERG. Patients have no systemic abnormality, but may be complicated with keratoconus or/and cataract. Family members of the probands were invited for a clinical and genetic assessment, if available. Written informed consent was obtained either from the participating individuals or their guardians. This study was approved by the Institutional Review Board of PUMCH, and adhered to the tenets of the Declaration of Helsinki and the Guidance on Sample Collection of Human Genetic Diseases by the Ministry of Public Health of China.

Clinical Evaluations

A full medical and family history was taken, and an ophthalmological examination was performed. Each patient underwent standard ophthalmic examination: best corrected visual acuity (BCVA) according to decimal Snellen charts, slit-lamp biomicroscopy, dilated indirect ophthalmoscopy, fundus photography, and visual field tests, if possible. The retinal structure was examined with optical coherence tomography (OCT; Topcon, Tokyo, Japan). Electroretinograms were performed (RetiPort ERG system; Roland Consult, Wiesbaden, Germany) using corneal "ERGjet" contact lens electrodes. The ERG protocol complied with the standards published by the International Society for Clinical Electrophysiology of Vision (ISCEV).

Library Preparation and Targeted Sequencing

Precapture Illumina libraries (Illumina, San Diego, CA, USA) were generated according to the manufacturer's standard protocol for genomic DNA library preparation. Briefly, 1 μg genomic DNA was fragmented to 200 to 500 base pairs (bp) long. The DNA fragments were end-repaired using polynucleotide kinase and Klenow. The 5′ ends of the DNA fragments were phosphorylated and a single adenine base was added to the 3′ ends using Klenow exo-nuclease. Illumina Y-shaped index adaptors were ligated to the ends, and the DNA fragments were PCR amplified for 8 cycles and fragments of 300 to 500 bp were isolated by bead purification. The precapture libraries were quantified using the PicoGreen fluorescence assay and their size distributions were examined by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A total of 48 precapture libraries (50 ng/library) was pooled together for one capture reaction. Agilent Sureselect Hybridization and Wash Kits were used for capture enrichment, following the standard manufacturer's protocol. Enriched libraries were sequenced on the Illumina HiSequation 2000 as 100-bp paired-end reads according to the manufacturer's protocols.

Bioinformatics Analysis of Sequencing Results

Raw sequencing reads were aligned to the human reference genome using BWA software and stored as .bam files.[22] The genome analysis tool kit (GATK) was used to refine the alignment.[23] Single nucleotide polymorphisms (SNPs) and Indels were called using Atlas2.[24] Variants were filtered against the 1000 genomes project/database, the Exome Variant Server (available in the public domain at http://evs.gs.washington.edu/EVS/), and the Baylor internal database, with a minor allele frequency cutoff of 0.5%.[25,26] Variants were annotated using ANNOVAR.[27] The pathogenicity of missense variants was predicted using dbNSFP, a program that compiles prediction scores from six prediction algorithms (SIFT,[28] Polyphen2,[29] LRT,[30] MutationTaster,[31] MutationAssessor,[32] and FATHMM[33]) and three conservation scores (Phylop, GERP++,[34] and Siphy[35,36]), was used to predict the pathogenicity of novel missense variants. The prediction of novel missense variants is listed in Supplementary Table S1.[37] If pathogenic mutations were found, further Sanger validation, segregation, and clinical reevaluation (if necessary) were performed.

Sanger Sequencing and Segregation Test

Human reference sequences were obtained from the UCSC genome browser (hg19; available in the public domain at https://genome.ucsc.edu/). RepeatMasker was used to identify the repetitive regions (available in the public domain at http://www.repeatmasker.org). Primers were designed by Primer3 (available in the public domain at http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Polymerase chain reaction amplicons (300–500 bp), which include the entire exon containing the mutation and at least 50 bp flanking regions, were sequenced on an ABI 3730xl capillary sequencing machine (Applied Biosystems, Waltham, MA, USA). All available family members were Sanger sequenced for the segregation analysis. The Sanger sequencing results were analyzed by Sequencher5.0 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistical Analysis

A 1-tailed Z-test was used for the analysis of mutation spectrum differences. A significance threshold of 0.05 was used.

Results

We collected DNA samples of 145 families who were diagnosed with LCA during their initial clinic visit. Among them, 120

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Table 1. Information of 145 LCA Families

	Family With 1 Pt	Family With 2 Pts	Family With 3 Pts
n	120	23	2
Families with parents available	110	22	2

families have only 1 LCA patient, 23 families have 2 LCA patients, and 2 families have 3 affected individuals. DNA samples of parents in 134 families were available for segregation testing (Table 1).

Mutation Screening in 145 LCA Families

Mutation Screening by Direct Sanger Sequencing. Initially, we performed direct Sanger sequencing of the coding regions in 7 known LCA disease genes (*AIPL1*, *CRB1*, *CRX*, *GUCY2D*, *LRAT*, *RDH12*, *RPE65*) in 81 families and successfully identified the pathogenic mutations for 28 families. In addition, we recruited 64 new families with at least one family member diagnosed with LCA. To reduce the cost and increase the solving rate, one proband from 117 families (53 + 64) was chosen for capture-sequencing in this cohort (Supplementary Table S2).

Mutation Screening Using Retinal Disease Gene Panel. Previously, our group has developed a capture panel of retinal disease genes, and applied this panel to a Chinese RP cohort and a European LCA cohort successfully.[7,38] In this study we expanded the panel to incorporate the coding exons and splicing sites of 163 known retinal disease genes and candidate retinal disease genes. This panel covers 1176 Mbp and 4405 exons, as well as the frequent intronic mutation c.2991+1655A>G in *CEP290* (Supplementary File S1). The DNA of the 117 probands from each family in this Chinese LCA cohort was capture-enriched and sent for next generation sequencing. On average, more than 1.6 M reads per sample were generated and mapped to the design regions. The mean sequencing coverage is ×114 with 75% of all the bases in the targeted region covered by at least 40 reads, indicating sufficient coverage for a high sensitivity of mutation detection.

Automatic Variant Filtering and Annotation. Our group has developed an automatic variant calling, filtering, and annotation pipeline as described in the methods section. All sequencing data from 117 probands were processed through this pipeline. After initially calling, an average of 3650 SNPs, and 378 small insertions and deletions (indels) were identified per sample. Considering that LCA is a rare Mendelian disease, polymorphisms that appear at a frequency higher than 0.5% (for recessive variants) or higher than 0.1% (for dominant variants) in any of the following databases were excluded from further analysis: the 1000 Genome database (builds 20110521 and 20101123), NHLBI Exome Sequencing database, NIEHS Exome Sequencing database, and our internal control databases. Therefore, an average of 11 SNPs and 6 Indels that are rare and lead to a protein coding change were identified in each sample.

Identification of Pathogenic Mutations in 145 LCA Families

We considered three factors when determining pathogenic mutations in each proband. First, we checked whether the mutations match the inheritance model. Second, the mutations identified by NGS were validated by direct Sanger sequencing. Third, if there are additional family members available, the mutations should cosegregate with the disease phenotypes. Based on this procedure, we identified pathogenic mutations in 111 of 145 families. Among the 111 families, 92 families carried mutations in known LCA disease genes and 5 families carried mutations not only in known LCA disease genes, but also other retinal disease genes. In addition, we found 14 families who carried mutations in non-LCA retinal disease genes.

Table 2. Classification of All Identified Putative Pathogenic Mutations

	Known	Novel	Total
Frameshift indels	3	31	34
Nonsense mutations	14	33	47
Fail-to-stop mutation	0	1	1
Splicing changes	0	17	17
Nonframeshift indels	1	3	4
Missense mutations	13	51	64
	31 (18.6%)	136 (81.4%)	167

Compound heterozygous mutations were the most common in this cohort and were identified in 81 families. A total of 26 families carried homozygous mutations in retinal disease genes. We also identified that three families with one heterozygous mutation have a dominant inheritance mode and one family with one hemizygous mutation shows an X-linked inheritance mode (Supplementary Table S3). A total of 167 distinct potentially pathogenic mutations was identified among 111 probands, including 136 novel mutations (81.4%). A wide spectrum of mutation types were identified, including 34 frameshift indels, 47 nonsense mutations, 1 fail-to-stop mutation, 17 splicing changes, 4 nonframeshift indels, and 64 missense mutations (Table 2; Supplementary Fig. S1).

The variants identified in this study can be classified into three categories based on the American College of Medical Genetics standards: category 1 has the highest confidence, and includes mutations previously reported to cause LCA as confirmed by a review of the primary publications; category 2 refers to novel deleterious mutations (nonsense mutations, splicing donor/accepter changes, frameshift indels, and fail-to-stop mutations) that are likely to cause protein loss-of-function (LOF); and category 3, which has the lowest confidence, refers to novel missense variants.[39,40]

Classification of Families Based on Clinical Significance of Mutations

Families Carrying Mutations in Known LCA Disease Genes. Consistent with the clinical diagnosis, the vast majority of mutations identified fall within known LCA disease genes. In

Table 3. The Probands in 12 Families Carrying Reported Mutations in Known LCA Disease Genes

Family ID	Gene	Type	Mutations
614	*CEP290*	Compound Heterozygous	c.1645C>T, p.R549X[48] c.4661_4663del, p.E1555del[49]
148	*CRX*	Heterozygous	c.571del, p.Y191MfsX3[50]
xh18513	*RDH12*	Homozygous	c.226G>A, p.G76R[51]
1550164	*RPE65*	Compound Heterozygous	c.1103A>G, p.Y368C[47] c.200T>G, p.L67R[51]
1663858	*CRB1*	Homozygous	c.3676G>T, p.G1226X[20]
1684042	*CRB1*	Homozygous	c.1756C>T, p.R526X[52]
1548568	*CRB1*	Homozygous	c.1756C>T, p.R526X[52]
163	*GUCY2D*	Homozygous	c.3020C>T, p.S1007L[53]
764	*GUCY2D*	Compound Heterozygous	c.1978C>T, p.R660X[54] c.2861T>C, p.L954P[55]
154	*IQCB1*	Homozygous	c.994C>T, p.R332X[11]
697	*IQCB1*	Homozygous	c.1090C>T, p.R364X[21]
87	*RPGRIP1*	Homozygous	c.534delG, p.E179SfsX11[20]

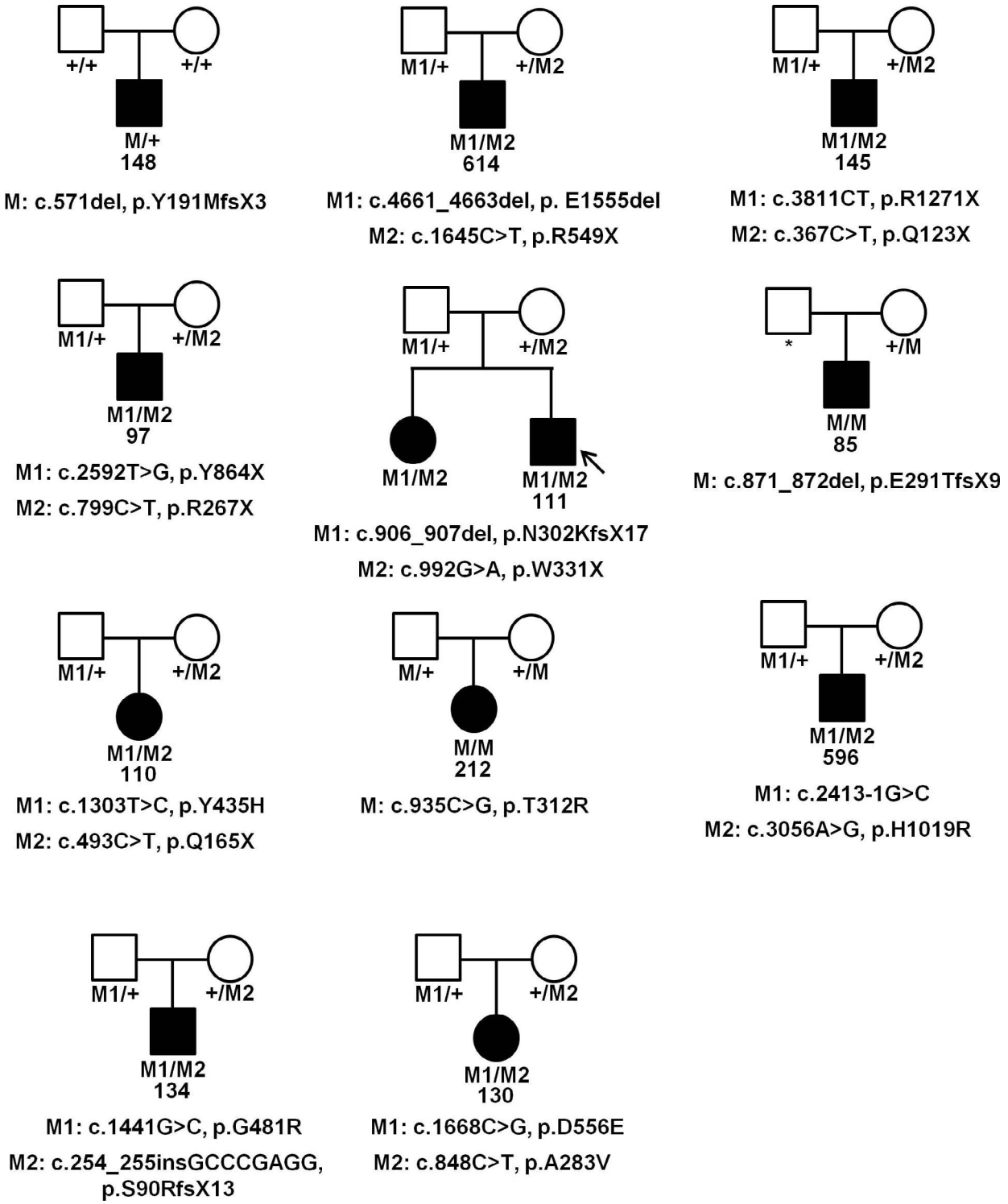

Figure 1. The segregation of mutations in families. *Black*: Affected. *White*: Unaffected. *Square*: Male. *Circle*: Female. *Star*: DNA not available. #Segregation test was done when DNA of family members were available. M, mutations.

this Chinese cohort, 92 families carry mutations in known LCA disease genes (Supplementary Table S4).

There are only 12 families that carry reported mutations (category 1) in known LCA disease genes (Table 3). Among them, eight families carry homozygous mutations, three carry compound heterozygous mutations, while one (148) carries a heterozygous frameshift mutation in *CRX*, which has been reported to cause autosomal dominant LCA. The segregation test suggests that neither parent of 148 carries the mutation, indicating that the mutation is de novo (Fig. 1). Similarly, a segregation test was performed for both parents of 614, and we found that the nonsense mutation was inherited from the mother and the nonframeshift deletion was inherited from the father, supporting the pathogenicity of the mutations (Fig. 1).

We also identified 42 families carrying categories 1 and 2 mutations in known LCA disease genes (Table 4). In total, 49

Table 4. The Probands in 42 Families Carrying Novel LOF Mutations in Known LCA Disease Genes

Family ID	Gene	Type	Mutations
389	*AIPL1*	Homozygous	c.421C>T, p.Q141X
394	*AIPL1*	Homozygous	c.421C>T, p.Q141X
3267585	*AIPL1*	Compound	c.421C>T, p.Q141X
		Heterozygous	c.834G>A, p.W278X[52]
89	*CEP290*	Compound	c.6870del, p.Q2290KfsX10
		Heterozygous	c.5246T>G, p.L1749X
137	*CEP290*	Compound	c.2586+1G>A
		Heterozygous	c.2484-2A>G
145	*CEP290*	Compound	c.3811C>T, p.R1271X
		Heterozygous	c.367C>T, p.Q123X
161	*CEP290*	Compound	c.7438T>C, p.X2480Q
		Heterozygous	c.1666del, p.I556FfsX17
499	*CEP290*	Compound	c.6870del, p.Q2291KfsX10
		Heterozygous	c.6012-2A>G
698	*CEP290*	Compound	c.1429C>T, p.R477X[56]
		Heterozygous	c.4438-1G>A
116	*CRB1*	Homozygous	c.2866G>T, p.G956X
118	*CRB1*	Compound	c.1008T>A, p.C336X
		Heterozygous	c.1831T>C, p.S611P[20]
125	*CRB1*	Compound	c.2172T>A, p.Y724X
		Heterozygous	c.2234C>T, p.T745M[57]
625	*CRB1*	Compound	c.1734_1752del, p.L580PfsX35
		Heterozygous	c.2234C>T, p.T745M[57]
727	*CRB1*	Compound	c.663T>A, p.C221X
		Heterozygous	c.1576C>T, p.R526X[52]
2211522	*CRB1*	Compound	c.2512A>T, p.K838X
		Heterozygous	c.3676G>T, p.G1226X[20]
1545586	*CRB1*	Compound	c.1831T>C, p.S611P[20]
		Heterozygous	c.2540_2541delTC, p.F847FfsX61
1636129	*CRB1*	Compound	c.652+2G>T
		Heterozygous	c.1831T>C, p.S611P[20]
157	*GUCY2D*	Compound	c.2113+1G>A
		Heterozygous	c.3020C>T, p.S1007L[53]
691	*GUCY2D*	Homozygous	c.2481C>G, p.Y827X
1686901	*GUCY2D*	Compound	c.82_83insC, p.R28PfsX291
		Heterozygous	c.2303G>A, p.R768Q[56]
1625818	*GUCY2D*	Compound	c.849C>A, p.Y283X[53]
		Heterozygous	c.1119_1120insGGTG, p.S374fsX74
141	*LCA5*	Compound	c.1466del, p.L489CfsX104
		Heterozygous	c.238C>T, p.R80X
85	*LCA5*	Homozygous	c.871_872del, p.E291TfsX9
76	*LCA5*	Homozygous	c.878del, p.D293VfsX2
1668163	*LCA5*	Compound	c.1207 C>T, p.Q403X[58]
		Heterozygous	c.35_38 delAAGA, p.Q12QfsX98
1665955	*LCA5*	Compound	c.1759_1760insAG, p.L587QfsX7
		Heterozygous	c.795T>G, p.Tyr265X[21]
132	*OTX2*	Heterozygous	c.23C>A, p.S8X
1679336	*CRX*	Heterozygous	c.421delT, p.S141PfsX46
152	*RD3*	Compound	c.451del, p.A151PfsX46
		Heterozygous	c.418_419insG, p.R140AfsX58
111	*RPE65*	Compound	c.992G>A, p.W331X
		Heterozygous	c.906_907del, p.N302KfsX17
103	*RPGRIP1*	Compound	c.534delG, p.E179SfsX11[20]
		Heterozygous	c.3565C>T, p.R1189X
115	*RPGRIP1*	Compound	c.1234C>T, p.Q412X
		Heterozygous	c.2009_2010insG, p.P672AfsX6
133	*RPGRIP1*	Compound	c.3560_3566del, p.R1189GfsX7[52]
		Heterozygous	c.3617+1G>A
153	*RPGRIP1*	Compound	c.534delG, p.E179SfsX11[20]
		Heterozygous	c.1225C>T, p.Q409X
77	*RPGRIP1*	Compound	c.490+1G>T
		Heterozygous	c.3560_3566del, p.R1189GfsX7[52]
79	*RPGRIP1*	Compound	c.490+1G>T
		Heterozygous	c.2554C>T, p.R852X[59]
93	*RPGRIP1*	Compound	c.367C>T, p.R123X
		Heterozygous	c.3618-5_c.3618-1del
97	*RPGRIP1*	Compound	c.2592T>G, p.Y864X
		Heterozygous	c.799C>T, p.R267X
558	*SPATA7*	Homozygous	c.1216-2A>T
86	*SPATA7*	Compound	c.340del, p.N114IfsX23
		Heterozygous	c.373-1G>A
121	*TULP1*	Compound	c.1444C>T, p.R482W[60]
		Heterozygous	c.1318C>T, p.R440X
74	*TULP1*	Homozygous	c.627del, p.S210QfsX27

distinct novel LOF mutations and 16 known mutations were identified in these families. Within this group, *CRB1* and *RPGRIP1* are the most frequently mutated genes, with variants in eight families, and *CEP290* is the third most frequently mutated gene, with variants in six families. Notably, family 389 and 394 share the same homozygous nonsense mutation in *AIPL1*. We further conducted cosegregation tests for some of the families. As a result, the nonsense mutation c.3811C>T (p.R1271X) in family 145 is paternally inherited, and the other nonsense mutation c.367C>T (p.Q123X) in family 145 is maternally inherited (Fig. 1). Similarly, the nonsense mutation c.799C>T (p.R267X) is heterozygous in the mother of the proband in family 97, while the other nonsense mutation c.2592T>G (p.Y864X) is heterozygous in the father of the proband in family 97 (Fig. 1). For family 111, the nonsense mutation is maternally inherited, and the deletion is paternally inherited. Moreover, the affected sister of the proband in family 111 also carries the same compound heterozygous mutations (Fig. 1). In addition, we confirmed that the deletion in the proband in family 85 is heterozygous in the mother (Fig. 1). We confirmed that the compound heterozygous frameshift indels in family 152 are in trans, given that the two adjacent mutations (34 bps away) are always on different NGS reads and are on different PCR amplicons in the Sanger sequencing results (data not shown). By checking whether two mutations are on different NGS reads, we also were able to confirm that the compound heterozygous mutations in families 125 and 133 are in trans (data not shown).

Additionally, we identified 38 families with one or more novel missense mutations in known LCA disease genes (Table 5). The gene *CRB1* is the most frequently mutated gene in this group, with 10 families carrying mutations in this gene. We conducted segregation tests for families 110, 114, 120, 212, and 596, and confirmed that the mutations are inherited from each parent, respectively. We also confirmed that the proband and the affected sibling in family 155 carry the same compound heterozygous mutations. By checking whether two mutations are on different NGS reads, we confirmed that the compound heterozygous mutations in families 600 and 138 are in trans (data not shown).

Families Carrying Mutations in Two Disease Genes. Interestingly, we identified five families carrying potentially pathogenic mutations in one known LCA gene and one other retinal disease gene (Table 6; Supplementary Table S4). Because it has been reported that the severity of disease can be modified or determined by more than one locus, we wanted to test this hypothesis in our cohort.[41,42]

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Table 5. The Probands in 38 Families Carrying One or More Novel Missense Mutations in Known LCA Disease Genes

Family ID	Gene	Type	Mutations
138	*CEP290*	Compound	c.6012-2A>G
		Heterozygous	c.6012G>C, p.R2004S
624	*CEP290*	Compound	c.1645C>T, p.R549X
		Heterozygous	c.1979A>T, p.E660V
142	*CRB1*	Compound	c.2371G>C, p.G791R
		Heterozygous	c.2681_2682insC, p.C896LfsX13
151	*CRB1*	Homozygous	c.3218T>C, p.F1073S
155	*CRB1*	Compound	c.3488G>T, p.C1163F
		Heterozygous	c.4005+2T>G
730	*CRB1*	Compound	c.98del, p.R33SfsX38
		Heterozygous	c.1997T>A, p.V666D
769	*CRB1*	Compound	c.2815T>G, p.C939G
		Heterozygous	c.3152G>A, p.W1051X[61]
799	*CRB1*	Compound	c.2416G>C, p.E806Q (1.58#)
		Heterozygous	c.2714G>A, p.R905Q
1688659	*CRB1*	Compound	c.2290C>T, p.R764C
		Heterozygous	c.3676G>T, p.G1226X[20]
1662591	*CRB1*	Compound	c.1756C>T, p.R526X[52]
		Heterozygous	c.3023T>C, p.L1008S
xh17695	*CRB1*	Compound	c.1202 G>A, p.C401Y
		Heterozygous	c.2462 C>G, p.T821R
1714763	*CRB1*	Compound	c.2815T>G, p.C939G
		Heterozygous	c.3676G>T, p.G1226X[20]
1686493	*GUCY2D*	Compound	c.2039 T>A, p.L680H
		Heterozygous	c.2804C>T, p.S935L
xh14070	*GUCY2D*	Compound	c.1070T>G, p.L357R
		Heterozygous	c.2008C>T, p.R670W
xh19814	*GUCY2D*	Compound	c.835G>A, p.D279N
		Heterozygous	c.3166C>G, p.L1056V
1558659	*GUCY2D*	Compound	c.935C>T, p.T312M[62]
		Heterozygous	c.995G>C, p.R332P
212	*GUCY2D*	Homozygous	c.935C>G, p.T312R
477	*GUCY2D*	Compound	c.2984G>T, p.R995L
		Heterozygous	c.2576C>T, p.P859L (1.765#)
596	*GUCY2D*	Compound	c.2413-1G>C
		Heterozygous	c.3056A>G, p.H1019R
623	*GUCY2D*	Compound	c.2576+1G>A
		Heterozygous	c.3037G>A, p.G1013R
120	*NMNAT1*	Compound	c.709C>T, p.R237C
		Heterozygous	c.713A>G, p.Y238C
94	*NMNAT1*	Compound	c.116-2A>G
		Heterozygous	c.634G>A, p.V212M (1.475#)
600	*NMNAT1*	Compound	c.713A>G, p.Y238C
		Heterozygous	c.721C>T, p.P241S
159	*RD3*	Homozygous	c.311T>C, p.L104P (1.59#)
1664583	*RDH12*	Compound	c.437T>A, p.V146D
		Heterozygous	c.601delT, p.C201AfsX77
1675310	*RDH12*	Compound	c.623T>A, p.V208E
		Heterozygous	c.524C>T, p.S175L[63]
1546091	*RDH12*	Compound	c.437T>A, p.V146D
		Heterozygous	c.721_723delTCC, p.S241del
114	*RDH12*	Compound	c.193C>T, p.R65X
		Heterozygous	c.437T>A, p.V146D
158	*RDH12*	Compound	c.505C>G, p.R169G
		Heterozygous	c.883C>T, p.R295X
1676734	*RPE65*	Compound	c.200T>G, p.L67R[51]
		Heterozygous	c.1590C>A, p.F530L
110	*RPE65*	Compound	c.1303T>C, p.Y435H
		Heterozygous	c.493C>T, p.Q165X
175	*RPE65*	Compound	c.1338G>T, p.R446S (0.535*)
		Heterozygous	c.200T>G, p.L67R[64]
113	*RPGRIP1*	Compound	c.2057_2058insA, p.D687RfsX16
		Heterozygous	c.G2398G>A, p.E800K
692	*SPATA7*	Compound	c.1183C>T, p.R395X[15]
		Heterozygous	c.1215G>T, p.E405D
98	*TULP1*	Compound	c.1153G>A, p.G385R
		Heterozygous	c.525_526insC, p.P176TfsX7
139	*TULP1*	Homozygous	c.1255C>T, p.R419W
1621712	*AIPL1*	Compound	c.421C>T, p.Q141X
		Heterozygous	c.572 T>C, p.L191P
1663520	*AIPL1*	Compound	c.152A>G, p.D51G
		Heterozygous	c.733_735delGAG, p.E245del

* When MutationAssessor_predition is low, MutationAssessor_score is shown.

The proband in family 171, who was a 38-year-old woman from a consanguineous marriage, carries a reported homozygous nonsense mutation *RPE65* and a novel homozygous nonsense mutation in *GPR179* (Fig. 2). The *GPR179* gene is a known retinal disease gene associated with recessive complete congenital stationary night blindness. She had poor visual function since infancy, which had progressively worsened. Visual acuity was light perception in both eyes. She had nyctalopia and exotropia. Her fundi showed macular dystrophy and attenuated retinal vessels with diffuse retinal mottling in both eyes (Fig. 3A). Optical coherence tomography showed macular atrophy without noticeable signal of the junction between inner and outer segments (IS/OS) under OCT scan (Fig. 3B). The proband in family 171 has a younger brother whose phenotype was milder. His BCVA was 0.2/0.3 and the fundi showed diffuse retinal mottling in the middle peripheral retina in both eyes, while the macular and retinal vessels appeared normal (Fig. 3C). Optical coherence tomography showed a relatively normal macula with weak IS/OS signal (Fig. 3D). His ERG was extinguished. Unlike his sister, who is the proband in family 171, this patient carries a heterozygous in *GPR179* instead of a homozygous mutation in this gene (Fig. 2). The proband in family 107, who is a 36-year-old woman, carries novel compound heterozygous mutations in known

Table 6. The Probands in Five Families Carrying Mutations in Two Genes

Family ID	Gene	Type	Mutations
107	*CRB1*	Compound	c.2172T>A, p.Y724X
		Heterozygous	c.3442T>C, p.C1148R
	TOPORS	Heterozygous	c.2944_2945del, p.V982RfsX2
171	*RPE65*	Homozygous	c.1543C>T, p.R515W[65]
	GPR179	Homozygous	c.709C>T, p.Q237X
497	*LCA5*	Homozygous	c.795T>G, p.Y265X[21]
	ALMS1	Homozygous	c.10235A>G, p.E3412G (1.1*)
571	*IQCB1*	Compound	c.1465C>T, p.R489X
		Heterozygous	c.1090C>T, p.R364X[21]
	PRPH2	Heterozygous	c.460A>C, p.K154Q (1.745*)
688	*AIPL1*	Compound	c.421C>T, p.Q141X,
		Heterozygous	c.325C>T, p.Q109X
	RHO	Heterozygous	c.310G>A, p.V104I[66]

* When MutationAssessor_predition is low, MutationAssessor_score is shown.

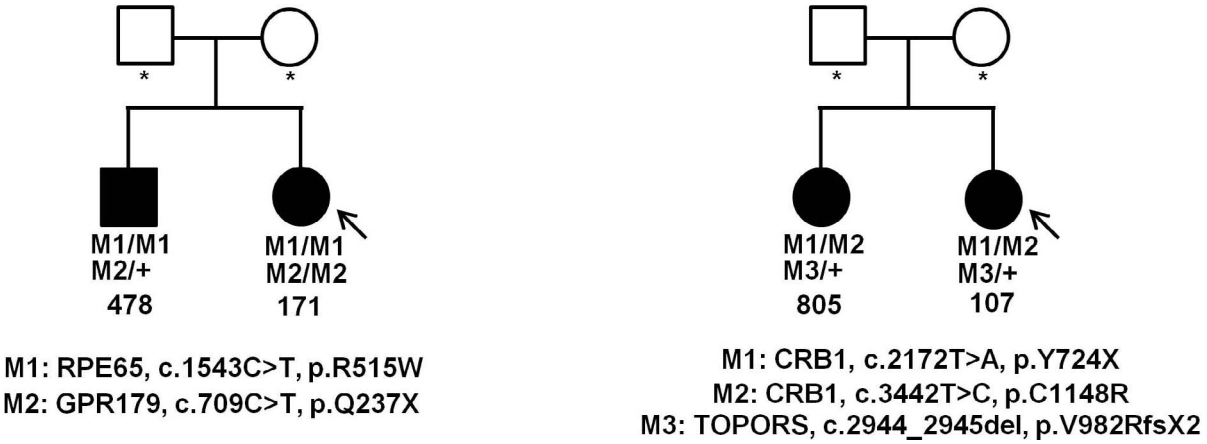

Figure 2. The segregation of mutations in two genes in families. *Black*: Affected. *White*: Unaffected. *Square*: Male. *Circle*: Female. *Star*: DNA not available.

LCA disease gene *CRB1*, and a novel heterozygous frameshift deletion in the gene *TOPORS*, which is associated with dominant RP. The same genotype also was confirmed in her monozygotic twin sister, patient 805. Both patients show more severe phenotypes than other LCA patients with *CRB1* mutations only. The proband complained of poor vision after birth and visual acuity was light perception in both eyes. The oculodigital phenomenon, nystagmus, and eyeball depression

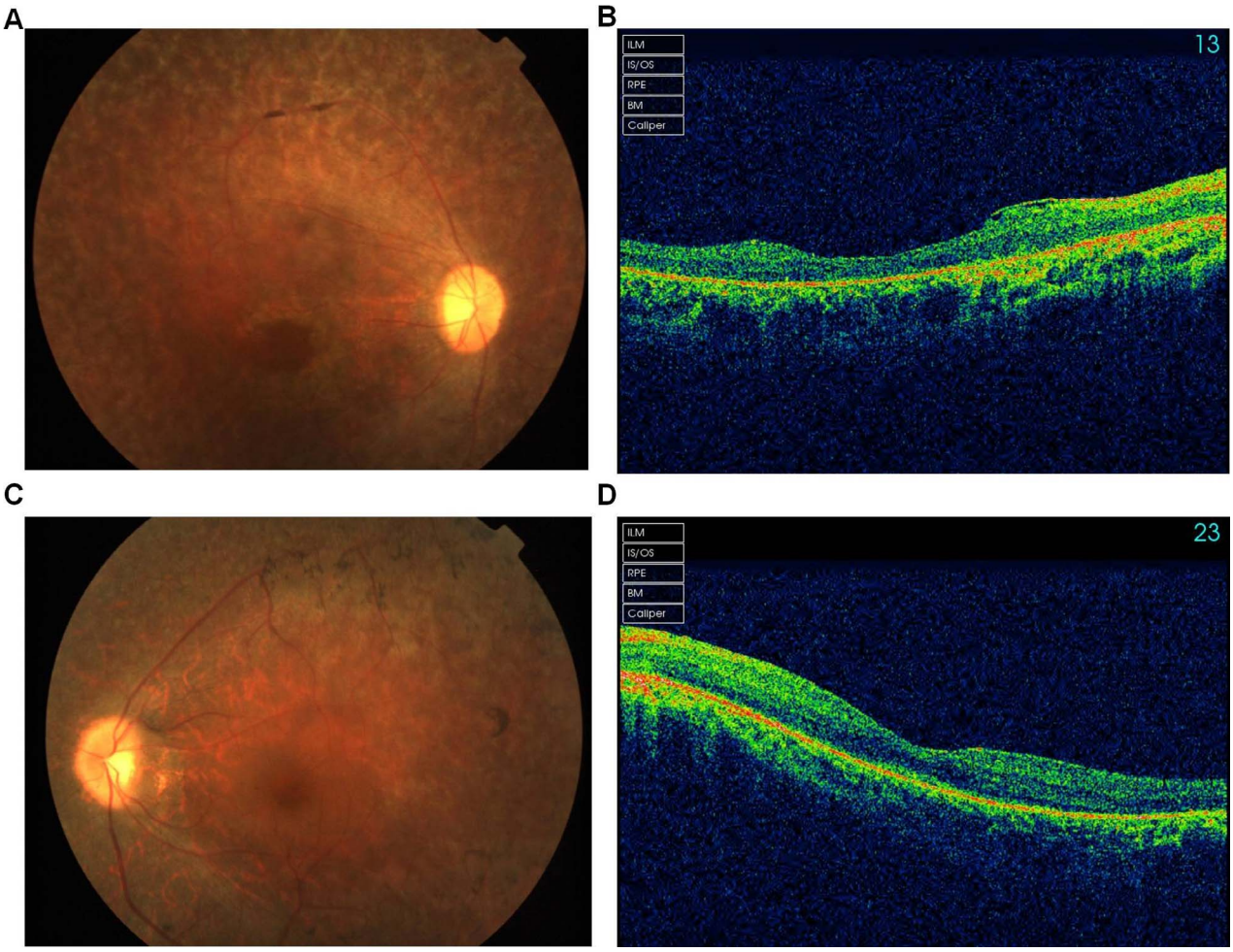

Figure 3. The phenotypes of the proband in family 171 and her affected brother 478. (**A**) The fundi of the proband in family171 showed macular dystrophy and attenuated retinal vessels with diffuse retinal mottling. (**B**) Optical coherence tomography of the proband in family 171 showed macular atrophy without noticeable signal of the junction between inner and outer segments. (**C**) The fundi of 478 showed diffuse retinal mottling in the middle peripheral retina. (**D**) Optical coherence tomography of 478 showed a relatively normal macular with weak IS/OS signal.

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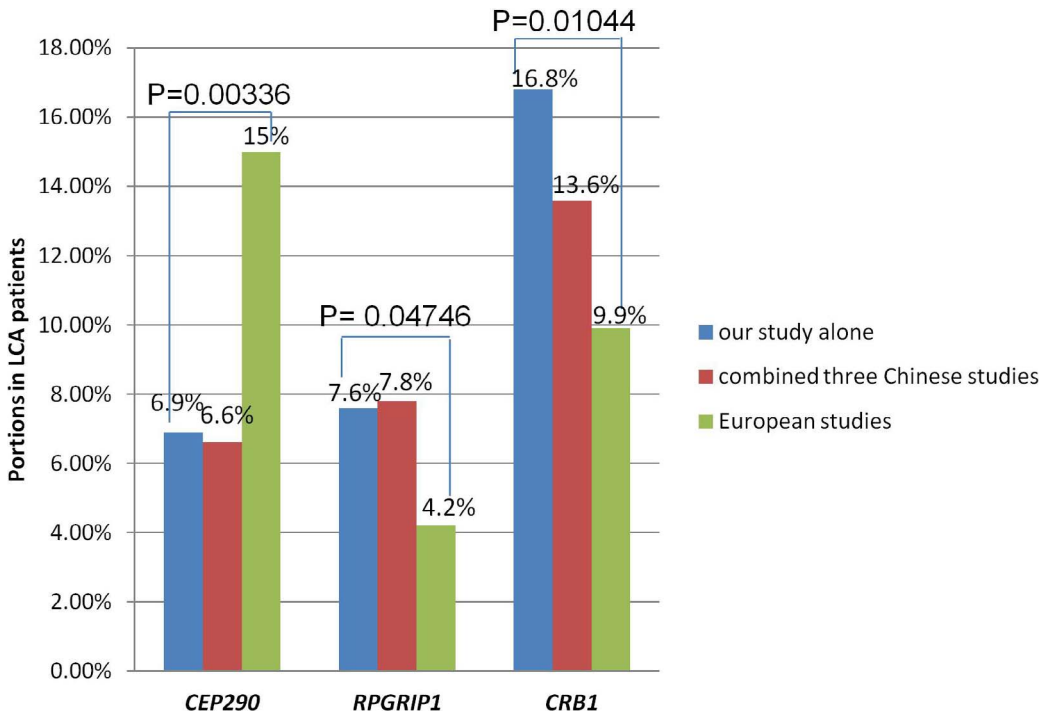

Figure 4. The percentage of LCA patients carrying mutations in *CEP290*, *RPGRIP1*, and *CRB1* in Chinese and European ancestry patients.

were present. Furthermore, she also displayed keratoconus with a cataract in the left eye. Dilated fundus examination of the right eye showed dense grayish white and grayish black pigments. Pronounced nystagmus and the cataract limit our ability to make detailed observations using fundus photography or OCT. The monozygotic twin sister of the proband had a similar manifestation.

Taken together, our results suggested that a combination of pathogenic mutations in two retinal disease genes increases the disease severity.

The Mutation Spectrum of Known LCA Disease Genes in the Chinese Population. The large number of families ($n = 145$) in our study allowed us to study the mutation spectrum of known LCA disease genes in Chinese LCA patients. Since 14 families carried pathogenic mutations in other retinal disease genes, we excluded them when we calculated the mutation frequency in 22 known LCA disease genes. As a result, we found that *CRB1* (16.8%, 22/131), *GUCY2D* (10.7%, 14/131), *RPGRIP1* (7.6%, 10/131), and *CEP290* (6.9%, 9/131), are the four most frequently mutated genes in our cohort (Supplementary Fig. S2A; Supplementary Table S5). Combining the results of our study and two previous studies in Chinese LCA patients, *CRB1* (13.6%, 33/243), *GUCY2D* (12.3%, 30/243), *RPGRIP1* (7.8%, 19/243), and *CEP290* (6.2%, 15/243) still are the four most frequently mutated genes in Chinese LCA patients (Supplementary Fig. S2B; Supplementary Table S5).[20,21] Comparing our results with previous estimations, the major mutation spectrum difference is that European ancestry LCA cases carry more mutations in *CEP290* (15.0%, 62/412, $Z = 2.7073$, $P = 0.00336$), and less mutations in *RPGRIP1* (4.2%, 37/872, $Z = -1.6666$, $P = 0.04746$) and *CRB1* (9.9%, 95/958, $Z = -2.3138$, $P = 0.01044$) than Chinese LCA cases (Fig. 4).[6] Indeed, none of the probands in our study carry the c.2991+1655A>G intronic mutation in *CEP290*, which is found frequently in European ancestry LCA patients, suggesting that this mutation has not been passed into the Chinese population during human evolution. On the other hand, a frequent deletion c.534delG (p.E179SfsX11) in *RPGRIP1* was identified in three LCA families in our study and two Chinese LCA patients reported previously.[20,21] To our knowledge, this deletion has never been reported in non-Chinese LCA patients. In addition, we identified a frequent novel nonsense mutation c.421C>T (p.Q141X) in *AIPL1* in five LCA families in our study. Our results revealed that the mutation spectrum of Chinese LCA patients is different from that of patients from other ethnicities.

Families Carrying Mutations in Non-LCA Retinal Disease Genes. Furthermore, 14 families carried mutations in other retinal disease genes. According to the same criteria mentioned above, we classified them into two groups.

A total of eight families, who carried novel LOF mutations in other retinal disease genes, belong to group 1 (Table 7). Five of them carried mutations in *ALMS1*, which causes Alström syndrome. The compound heterozygous mutations in *ABCA4* cosegregated with the phenotype in family 104. The male proband in family 100 carries a hemizygous novel splicing change in *RPGR*, while his mother carries the heterozygous mutation and his father is homozygous wild-type.

Group 2 has six families carrying novel missense mutations in other retinal disease genes (Table 8). For example, the proband in family 134 carries an 8 bp insertion (c.254_255insGCCCGAGG, p.S90RfsX13) in *KCNV2* which causes a frameshift and a missense mutation (c.1441G>C, p.G481R) which changes glycine into arginine and is predicted to be damaging by all in silico algorithms (Supplementary Table S2). We performed cosegregation tests and confirmed that the compound heterozygous mutations in families 134 and 130 are inherited from each parent separately and are thus in trans.

Clinical Re-evaluation for Patients Carrying Mutations in Non-LCA Retinal Disease Genes

Interestingly, we observed 14 (8 + 6) families carrying mutations in other retinal disease genes. There are two possible explanations for this observation: first, the initial

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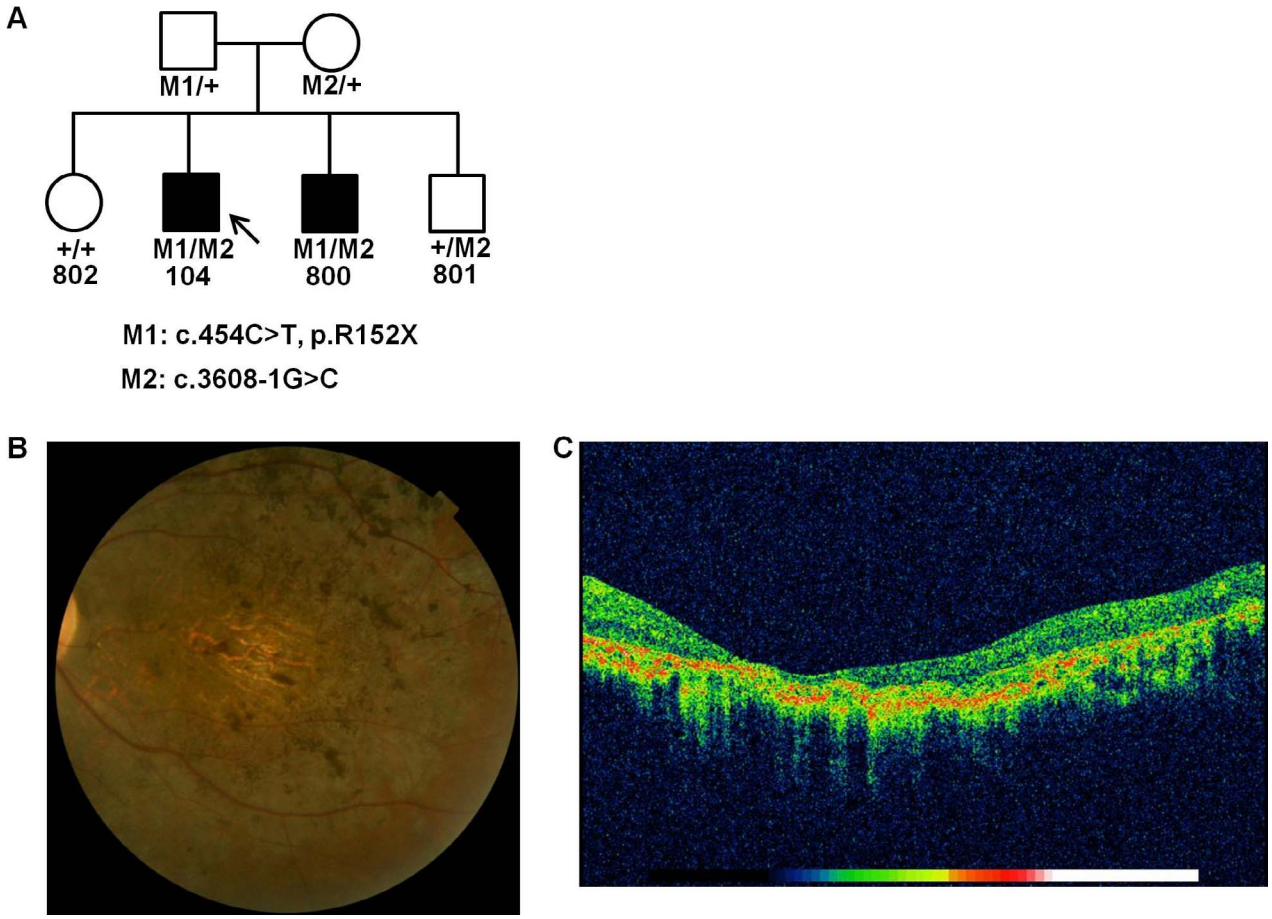

Figure 5. The pedigree and the phenotype of family 104. (**A**) The pedigree of family 104. (**B**) Dilated fundus examination of the proband in family 104 showed obvious macular atrophy and scattered bone spicule pigmentation. (**C**) Optical coherence tomography indicated severe macular atrophy.

clinical diagnosis is correct and there are novel genotype-phenotype correlations due to the different nature of alleles and different genetic backgrounds; second, the initial clinical diagnosis is incomplete due to the difficulty assigning clinical diagnoses at the time of the initial visit. To test two possibilities, we revisited 13 out of 14 families for their clinical phenotypes.

Molecular Diagnosis Helps to Refine the Initial Clinical Diagnosis. After revisiting, we refined the clinical diagnosis of 10 families based on their molecular diagnosis. For example, the proband in family 104 carries a novel compound heterozygous nonsense mutation and a splicing change in gene *ABCA4*, which is associated with recessive Stargardt disease, macular dystrophy, RP, and cone–rod dystrophy. The mutations segregated with the phenotypes in the family of 104, given that each parent only carries one of the two mutations, the affected sibling 800 carries the same compound heterozygous mutations, and the two unaffected siblings are either wild type or heterozygous for one mutation (Fig. 5A). After revisiting this family we confirmed the clinical diagnosis of LCA for the proband in family 104, but modified the disease type to cone-rod dystrophy for the younger brother 800. The proband in family 104 complained about poor vision with night vision problems at childhood, with a BCVA approximately 0.1 in both eyes during school age. His visual acuity at examination was hand motion/light perception (HM/LP) with a nonrecordable ERG. He had exotropia in both eyes. Dilated fundus examination showed obvious macular atrophy and scattered bone spicule pigmentation (Fig. 5B). After reviewing his fundus photo early in life, we found that the macular change occurred earlier than the peripheral area. Optical coherence tomography indicated severe macular atrophy (Fig. 5C). The younger brother 800 had a similar, but milder condition. His visual acuity was 0.03/0.05. The ERG was severely decreased, but still had recordable cone and rod responses. Therefore, the younger brother had cone–rod dystrophy, which has been associated previously with *ABCA4* mutations.[43]

In another example, the proband in family 88 carries novel homozygous frameshift insertions in the first exon of gene *CNNM4*, which has been associated previously with cone-rod dystrophy with amelogenesis imperfecta (Table 7).[44] After revisiting we, indeed, identified features of cone–rod dystrophy and amelogenesis imperfecta (Fig. 6). The 8-year-old girl complained of poor vision since she was 6 years old. During the initial visit, her BCVA was 0.3 in both eyes, and has now decreased to 0.1 in both eyes. Nystagmus, esotropia, and hyperopia were present. Dilated fundus examination showed macular atrophy with yellow appearance and scattered bone spicule pigmentation (Fig. 6A). Optical coherence tomography indicated severe macular atrophy and the ERG was extinguished.

Based on their clinical phenotype during revisiting, we modified the disease to RP for families 82 and 100, and to Alström syndrome for families 123, 126, 128, 162, 165, and 695. For example, the proband in family 162 was a 13-year-old boy who was diagnosed with LCA at his first evaluation. Now,

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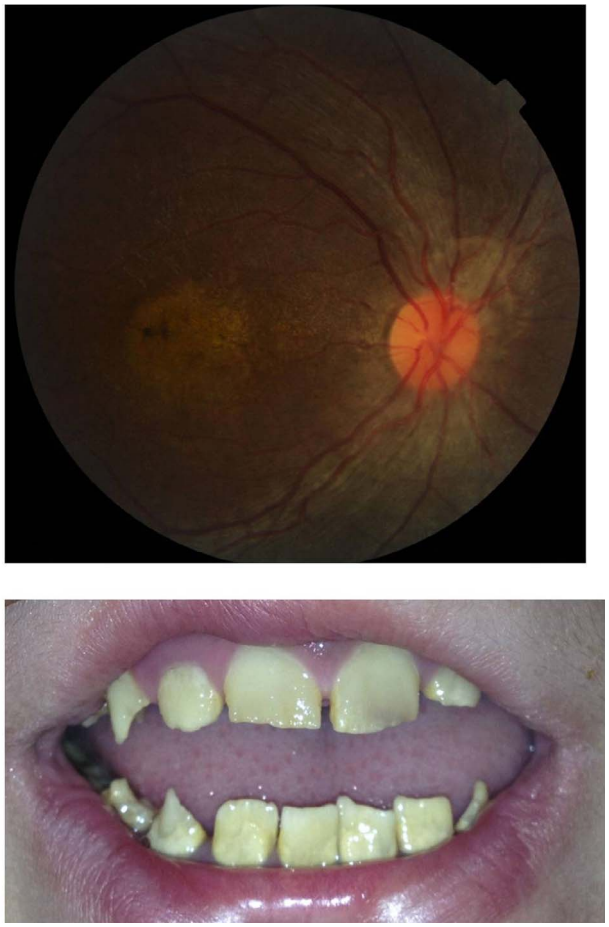

FIGURE 6. The phenotype of the proband in family 88. (**A**) Dilated fundus examination showed macular atrophy with yellow appearance and scattered bone spicule pigmentation. (**B**) Amelogenesis imperfecta.

he has the clinical phenotype of Alstrom syndrome, including type 2 diabetes, hepatic dysfunction, renal dysfunction, and acanthosis nigricans.

"LCA-Like" Phenotypes in Three Families. Due to the limited clinical information, the disease type of three families with mutations in other retinal disease genes could be either LCA, or an extreme spectra of other retinal diseases. For example, the proband in family 134, who was a 2-year-old boy, carries a frameshift insertion and a missense mutation predicted to be damaging by all in silico algorithms in the gene *KCNV2*, which is associated with recessive cone dystrophy with a supernormal rod electroretinogram (Supplementary Table S2). According to a segregation test, the mutations are in trans, being inherited one from each parent (Fig. 1). He had photophobia and nystagmus. Dilated fundus examination and OCT showed bilateral macular coloboma-like lesion and his ERG was severely decreased (Figs. 7A, 7B). However, we did not observe cone dystrophy with supernormal rod electroretinogram. Anterior segment examination showed normal results. Therefore, his phenotype does not fit the typical clinical presentations associated with *KCNV2*, but are more like LCA. Similarly, the female proband in family 130 carries compound heterozygous missense mutations in the gene *INPP5E*, which is known to be associated with Joubert syndrome that is characterized by neurological defects, retinal dystrophy, and renal anomalies. The two mutations are predicted to be pathogenic by in silico algorithms and are inherited one from each parent (Fig. 1; Supplementary Table S1). Her visual function had been poor from infancy with nystagmus and nyctalopia, and had progressively worsened. Visual acuity was 0.03 in both eyes. Her fundi showed attenuated retinal vessels with pepper-salt and bone spicule pigmentation in both eyes (Fig. 7C). Optical coherence tomography showed a thinned retina with a preserved signal of the IS/OS in the central fovea (Fig. 7D). However, we did not observe any syndromic features in addition to the LCA phenotype. Additionally, we did not observe hearing loss for the proband in family 728, who carries potentially pathogenic mutations in the Usher syndrome gene *PCDH15*. Our results emphasized the fact that patients diagnosed with LCA may carry mutations in other retinal disease genes.

DISCUSSION

We reported here the use of a capture panel-based NGS method for the molecular diagnosis of a large set of Chinese LCA patients. In total, 145 LCA families were recruited in this study, making it the largest Asian LCA cohort so far to our knowledge. Furthermore, we not only examined known LCA disease genes, but also screened for mutations in other known retinal disease genes. Together with other recent studies, we gained insight into the genetic basis of Chinese LCA patients, which previously were less studied.[20,21]

Because of the clinical and genetic overlap between LCA and other retinal diseases, it is necessary to perform a comprehensive mutation screen in probands to increase the solving rate. Indeed, we have identified that 97 + 14 (76.6%, 111/145) probands carry pathogenic mutations in one of the 163 known retinal disease genes. This solving rate is comparable to 75%, which is the estimated proportion that can be explained by known LCA disease genes.[6–10] However, if we restricted our search to the 22 known LCA disease genes only, the solving rate in this Chinese LCA cohort would be 66.9%, which is lower than that in the European population. This suggests that overall the genetic basis of Chinese LCA patients is unique, which could be explained by three reasons: First, Chinese patients may carry unique mutations in known LCA disease genes. For example, Chinese patients may carry founder intronic mutations or structural variants that could not be detected by our method and are absent in European ancestry LCA patients, similar to the founder intronic mutation in *CEP290* in European ancestry LCA patients. Second, some patients may carry mutations in other retinal disease genes due to the complexity of retinal diseases. Indeed, by analyzing mutations in other known disease genes, we found pathogenic mutations for an additional 14 families in this cohort. Third, there are novel LCA disease genes that are mutated in Chinese LCA patients and are less studied. We can potentially perform WES for the unsolved cases and look for novel LCA disease genes.

Comparing the mutant alleles found in our cohort to those reported in the literature, we found a large number of novel alleles were obtained in this study, suggesting that the mutation spectra of Chinese and European ancestry LCA patients are different. First, Chinese LCA patients carry more mutations in *RPGRIP1* (7.8% vs. 4.2%).[6] This could be explained partly by frequent founder mutations, which uniquely appeared in Chinese LCA patients. Indeed, we identified a frequent deletion c.534delG (p.E179SfsX11) in *RPGRIP1* and a frequent novel nonsense mutation c.421C>T (p.Q141X) in *AIPL1* that have not been reported in other ethnicities. Second, Chinese LCA patients carry fewer mutations in *CEP290* than European ancestry LCA patients (6.6% vs. 15.0%). Actually, a large proportion of European ancestry LCA patients carry a founder intronic mutation in *CEP290*, while in our study we did not find any Chinese LCA patients carrying this mutation.[6,45] These

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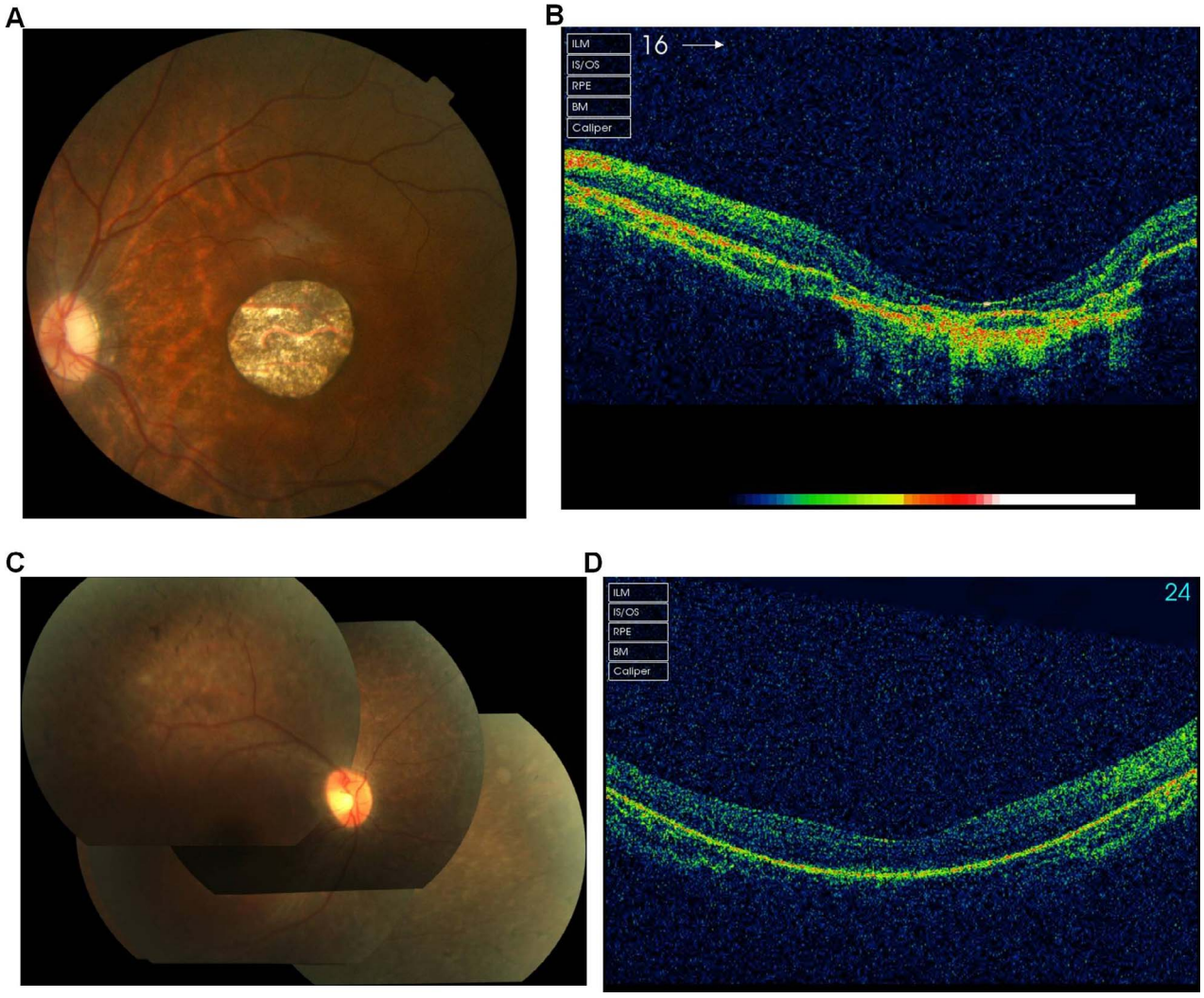

Figure 7. The phenotypes of the proband in families 134 and 130. (**A**) Dilated fundus examination of the proband in family 134 showed bilateral macular coloboma-like lesion. (**B**) Optical coherence tomography of the proband in family 134 indicated severe macular atrophy. (**C**) Fundi of the proband in family 130 showed attenuated retinal vessels with pepper-salt and bone spicule pigmentation. (**D**) Optical coherence tomography of the proband in family 130 showed thinned retina with preserved signal of IS/OS in the central fovea.

Table 7. The Probands in Eight Families Carrying Novel LOF Mutations in Non-LCA Retinal Disease Genes

Family ID	Gene	Type	Mutations
104	*ABCA4*	Compound Heterozygous	c.3608-1G>C c.454C>T, p.R152X
123	*ALMS1*	Homozygous	c.11110_11128del, p.R3704LfsX11
128	*ALMS1*	Compound Heterozygous	c.2064del, p.D689IfsX4 c.9441_9442insAATA, p.S3149KfsX2
162	*ALMS1*	Homozygous	c.2084C>A, p.S695X
165	*ALMS1*	Compound Heterozygous	c.805C>T, p.R269X c.3181C>T, p.Q1061X
695	*ALMS1*	Compound Heterozygous	c.5411del, p.Y1805TfsX23 c.5692_5695del, p.E1899RfsX18
88	*CNNM4*	Homozygous	c.896_897insT, p.A300CfsX22
100	*RPGR*	Hemizygous	c.310+1G>A

Table 8. The Probands in Six Families Carrying Novel Missense Mutations in Non-LCA Retinal Disease Genes

Family ID	Gene	Type	Mutations
126	*ALMS1*	Compound Heterozygous	c.7396G>T, p.E2466 c.9643T>A, p.S3215T (1.525*)
130	*INPP5E*	Compound Heterozygous	c.1668C>G, p.D556E c.848C>T, p.A283V
134	*KCNV2*	Compound Heterozygous	c.254_255insGCCCGAGG, p.S90RfsX13 c.1441G>C, p.G481R
689	*NPHP1*	Compound Heterozygous	c.2098G>C, p.G700R c.1754A>G, p.Y585C
728	*PCDH15*	Compound Heterozygous	c.5308_5313del, p.A1770_P1771del c.2899C>T, p.R967C
82	*RP2*	Homozygous	c.751A>G, p.R251G

* When MutationAssessor_predition is low, MutationAssessor_score is shown.

findings are important, because they help us to decide which genes should have a higher priority for screening during the molecular diagnosis of LCA patients from different ethnicities.

In our study, we identified five LCA families with pathogenic mutations in two retinal disease genes. Among them, we confirmed that the probands in families 171 and 107, indeed, showed much more severe phenotypes. Similar to this finding, it has been reported that LCA patients carrying a third allele in a second gene can present a more severe phenotype than family members who do not carry the third allele.[46,47] In our case, each gene alone carries pathogenic mutations that fit the inheritance pattern and are expected to be disease causing. Therefore, it is not surprising that the probands in families 171 and 107 with pathogenic mutations in two genes showed much more severe phenotypes. It also is worth mentioning that this scenario can only be identified when we sequence a larger set of retinal disease genes together. Our results emphasize the complexity of Mendelian diseases in the postgenome era.

In total, 10 families were reclassified to related retinal diseases that are consistent with their molecular diagnosis. Indeed, classifying a retinal disease patient as a certain disease type at a relatively early age is difficult. First, some systemic symptoms may not present at an early age. Second, some patients cannot express their complaint clearly, cannot cooperate with physicians, or are physically incompatible with an affiliated examination, such as an ERG. In the case where the proband in family 88 carries homozygous frameshift indels in the gene *CNNM4*, amelogenesis imperfecta was not noticed during the first evaluation, since the proband did not cooperate with the doctor. On the other hand, the clinical presentations of LCA and many other retinal diseases overlap.[6] In our study, patient 800 from family 104 was initially diagnosed with LCA, but after revisiting we found his phenotype was more similar to cone–rod dystrophy, because his ERG showed severely decreased, but still recordable, cone and rod responses. Therefore, our results suggested that the initial clinical diagnosis may not be perfect and molecular diagnosis could be a useful tool to refine or change the clinical diagnosis.

In summary, we successfully identified pathogenic mutations in 111 Chinese LCA families among a 145-LCA Chinese cohort. Together with data from previous studies, we have obtained a mutation spectrum for the Han Chinese population. Our study also highlighted that a comprehensive molecular diagnosis can facilitate a more accurate clinical diagnosis, which can lead to gene-specific treatments in the future.

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