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Detecting genetic variations in hereditary retinal dystrophies with next-generation sequencing technology

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Purpose: To identify pathogenic mutations responsible for retinal dystrophies (RDs) in three unrelated Chinese families.
Methods: Three probands from unrelated families with RDs were recruited. Genomic DNA prepared from leukocytes was analyzed using gene chip–based next-generation sequencing (NGS) to capture and sequence all of the exons of 100 known RD-associated genes. Candidate variants were validated with PCR and Sanger sequencing in the respective families. Thorough ophthalmic examinations including best-corrected visual acuity, funduscopic examination, and full-field electroretinograms were performed in the affected individuals.
Results: We successfully identified causative mutations in patients from the Chinese families with RDs: the known mutation *IMPDH1* c.942_944delGAA in a family with retinitis pigmentosa, the novel mutation *ABCA4* c.1924T>A in a family with Stargardt disease, and the novel mutation *NMNAT1* c.272A>G and known mutation *NMNAT1* c.196C>T in a family with Leber congenital amaurosis. All variations segregated with the disease phenotypes in the respective families and were absent from ethnically matched control chromosomes. Prediction analysis demonstrated the two novel missense mutations might be damaging.
Conclusions: The results strongly suggested these mutations were responsible for different RD phenotypes in the Chinese families. NGS technology provides an accurate and economic method for identifying causative genes for RDs.

Hereditary retinal dystrophies (RDs) are a large group of clinically and genetically heterogeneous retinal diseases that constitute a major cause of vision handicap in the global population [1]. They are typically characterized by the progressive loss of rod and cone photoreceptor cells often leading to severe blindness. There are similar fundus appearances among different RDs, including retinal pigment epithelium (RPE) changes, attenuated retinal vessels, and waxy optic disc. To date, more than 100 genes associated with various RDs have been identified and partially characterized at the genetic and molecular level (RetNet). These gene functions involved diverse biologic pathways, including phototransduction, visual cycle, photoreceptor structure and morphogenesis, cell adhesion, cellular metabolism, vesicle and protein trafficking, synaptic function, cilium structure and transport, ion and small molecular transport, chaperones, RNA splicing, transcription factors associated with photoreceptor development, protein folding and subunit assembly, and posttranslational protein modification, among others. Some RDs associated with different pathogenic genes show the same phenotypes while patients with RDs with the same causative gene can present different phenotypes. Therefore, accurate and comprehensive molecular diagnosis is critical for confirming the clinical diagnosis, providing a disease prognosis, and in the long run providing the basis for novel therapeutic approaches and personalized medicine. However, molecular diagnosis of RDs is made challenging by the large number of disease genes. Recently, next-generation sequencing (NGS) technology has provided a high-throughput and cost-effective method for identifying causative genes for RDs that could sequence the candidate gene rapidly and accurately [2-4]. The causative mutations of approximately 50% to 80% of RD cases could be detected successfully using this method [5-7].

In this study, we identified four mutations in three unrelated Chinese pedigrees diagnosed with RDs: the known mutation *IMPDH1* c.942_944delGAA in a family with autosomal dominant retinitis pigmentosa (adRP), the novel mutation *ABCA4* c.1924T>A in a family with Stargardt disease (STGD), and the novel mutation *NMNAT1* c.272A>G and the known mutation *NMNAT1* c.196C>T in a family with Leber congenital amaurosis (LCA). These variants were identified with the combined approach of target region sequencing–based NGS and candidate mutations validation with Sanger

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sequencing. They segregated with the disease phenotype in the respective families and were not detected in the normal controls database.

METHODS

Pedigrees and controls: All three pedigrees (Family-012, Family-024, and Family-035) described in this study were from Sichuan province in China. A detailed medical history was obtained by interviewing family members. The patients from the respective families underwent comprehensive ophthalmological examinations at the Southwest Eye Hospital, including best-corrected visual acuity (BCVA), dilated fundoscopic indirect ophthalmoscopy, and full-field electroretinograms (ERGs). ERGs were also performed and recorded according to the standards of the International Society for Clinical Electrophysiology (ISCEV). The normal controls database was from BGI, including 840 unrelated individuals, who were more than 50 years old and had no symptoms of night blindness, vision reduction, and any personal or family history of known inheritance disease. This research followed the tenets of the Declaration of Helsinki and received approval from the Ethic Committee of Southwest Hospital. Informed consent was obtained from all participants included in this study.

Targeted region capture and next-generation sequencing: A custom-made capture array (Roche NimbleGen, Madison, WI), from Beijing Genomics Institute-ShenZhen, was designed to capture the coded exons of the 100 RD genes (Appendix 1). Genomic DNA of the probands (Family-012 III:4, Family-024II:6, and Family-035II:2) was extracted from peripheral blood according to the manufacturer's standard procedure using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). Then the genomic DNA was fragmented to 200–300 bp by Covaris S2 (Woburn, MA). The library was pooled and hybridized to the custom capture array for 72 h at 42 °C. After hybridization, the array was washed and eluted according to the manufacturer's instructions (Roche NimbleGen). The captured library was sequenced on Illumina HiSeq2000 analyzers for 90 cycles per read to generate paired-end reads (following the manufacturer's standard sequencing protocols). Image analysis and base calling were performed using the Illumina Pipeline to generate raw data.

Variant identification and validation: To detect the pathogenic variants of the probands, we applied filtering criteria to generate clean reads (with a length of 90 bp) for further analysis, and then aligned the clean reads against the human genome reference from the NCBI database (version hg19) using the BWA (Burrows Wheeler Aligner) Multi-Vision software package. Single-nucleotide variants (SNVs) and indels were identified using SOAPsnp software and the Samtools Indel Genotyper, respectively. All SNVs and indels were determined using the NCBI dbSNP, HapMap project, 1000 Genome Project, and the database of healthy Chinese adults from BGI.

The candidate variations in known causative RD genes were validated with PCR and Sanger sequencing. PCR primers were designed via Primer6.0 as shown in Table 1. The PCR reactions were performed as follows: denaturation at 94 °C for 3min, followed by 30 cycles of 94 °C for 30s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1min, ending with a final extension step of 72 °C for 5min. The PCR products were sequenced using a BigDye terminator v3.1 cycle sequencing kit (ABI, Foster City, CA) and analyzed on an ABI 3730XL Genetic Analyzer.

Prediction analysis: To predict the influence of these variants on protein function, we analyzed evolutionary conservation of these mutations using the ClustalW tool and examined the possible impact of the amino acid substitution with SIFT and PolyPhen tools available online.

RESULTS

Clinical findings : The pedigree Family-012 was consistent with autosomal dominant inheritance (Figure 1). There were six affected individuals out of 20 members in this four-generation Chinese family. All the patients in the family had visual disabilities but no history of systemic abnormalities. Night blindness was always the presenting symptom, and the age of onset was about 12 years old (mean age of onset=11.6 years). Affected members also reported palpable peripheral visual field loss and mild visual acuity decline in their 30s.

Table 1. Primers used for potential pathogenic mutations amplification.

Mutation	Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)
c.942_944delGAA	*IMPDH1*	caccaagcctgcttcatttc	gggtcctcataaacctccac	310
c.1924T>A	*ABCA4*	ccatcctttgtccctctgtg	actccagcacccccattag	261
c.272A>G	*NMNAT1*	ttgatcgtaatatttcctgt	aagccgcttagccatttaca	308
c.196C>T	*NMNAT1*	ttgatcgtaatatttcctgt	aagccgcttagccatttaca	308

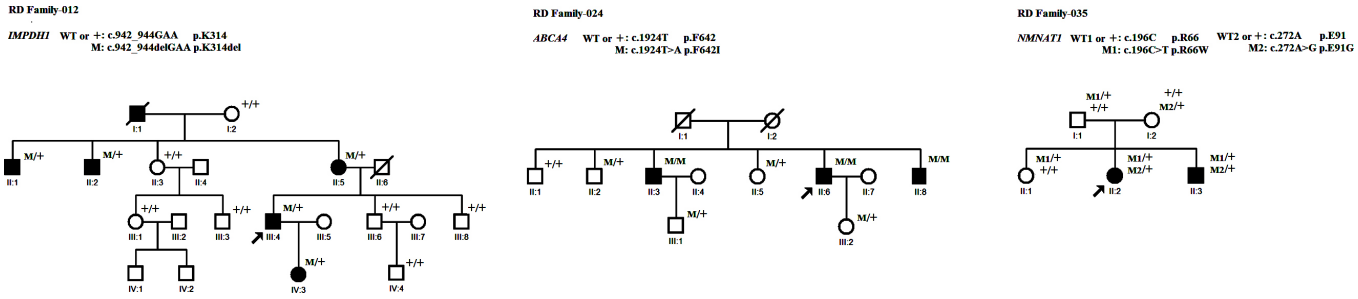

Figure 1. The pedigrees of the families with retinal dystrophies. Squares and circles indicate men and women, respectively. The dark symbols represent the affected members, and the slashed symbols denote that the subject is deceased. The patient above the arrow is the proband. WT or +: wild-type, M: mutation.

Funduscopic examinations revealed similar clinical manifestations among affected individuals, including waxy-pale discs, attenuation of retinal arterioles, bone spicule-like pigmentation in the periphery, and atrophy of the RPE (Figure 2). ERGs showed non-recordable responses (Figure 3).

Family-024 had autosomal recessive macular dystrophy (Figure 1). Affected individuals typically experienced significant reduction in central visual acuity in their second decade of life. The patients also showed a delay in dark adaptation in their 40s. Fundus photographs showed extensive atrophic-appearing RPE changes and pigmentation in the posterior pole of the retina (Figure 2). Abnormal cone function (assessed with light-adapted 30 Hz and light-adapted 3.0) and rod ERG abnormality (assessed using dark-adapted 0.01 to dark-adapted 10.0) were observed on ERG (Figure 3).

Family-035 was a small recessive inheritance family with only two affected individuals (Figure 1). They had horizontal nystagmus and poor vision but otherwise normal physical and mental health at 3 months of age. At 6–8 years of age, ophthalmological examinations showed that BCVA were about HM/30 cm in the both eyes, severe central atrophy with an appearance of macular coloboma. The remainder of the retina presented pigmentary changes, attenuated retinal blood vessels, and optic disc pallor (Figure 2). All ERGs responses were nearly extinct (Figure 3).

Identification of candidate mutations: Approximately 43.3 kb of exons and adjacent intronic regions of the 100 RD genes for the respective proband were captured and sequenced in our study. For the 100 RD genes, the average coverage is approximately 98.1%, and the average median-depth is 479×. On average, 100% of base pairs with N200× coverage were successfully detected indicating high capabilities for identifying variants.

In the respective probands of the three pedigrees, we first identified SNVs and indels in the coding regions and introns that may affect splicing. Since RDs are rare disorders but have a clear phenotype, the probability of patients with RDs sharing casual mutations with the healthy population is low.

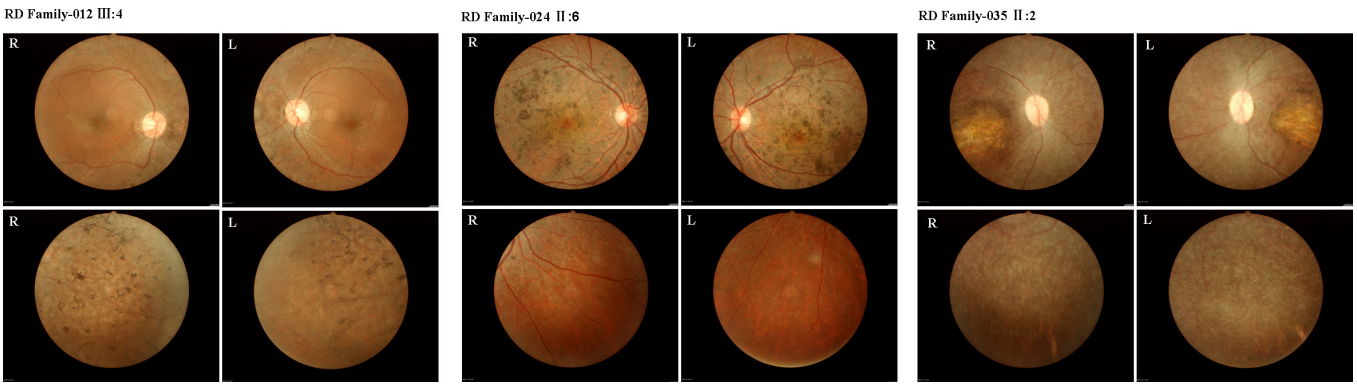

Figure 2. Bilateral fundus photography of the probands from the respective families with retinal dystrophies. Bilateral fundus photography shows attenuation of retinal arterioles, atrophy of the retinal pigment epithelium, and the waxy-pale disc of both eyes in all fundus photograph. Bone spicule-like pigmentation was found in the periphery retina of patient III:4 in Family-012, sheet pigmentation was found in the posterior pole retina of patient II:6 in Family-024, and macular coloboma was found in patient II:2 in Family-035.

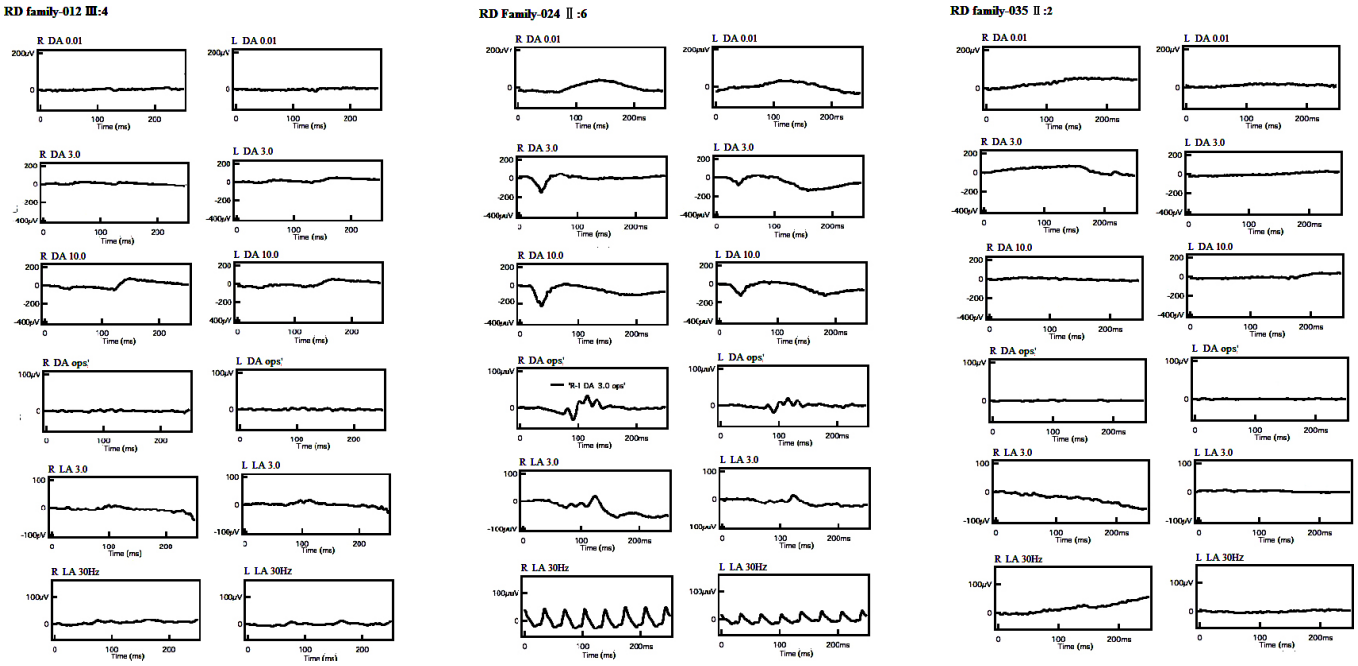

Figure 3. Full-field electroretinograms from the three probands in respective families with retinal dystrophies (patient III:4 in Family-012, patient II:6 in Family-024, and patient II:2 in Family-035). Patient III:4 in Family-012 and patient II:2 in Family-035 demonstrated undetectable ERG. Patient II:6 in Family-024 had delayed and reduced responses in dark-adapted (DA) 0.01, DA 10.0, light-adapted (LA) 3.0, and LA 30 Hz.

Therefore, we compared these variants in the proband with that of dbSNP132, HapMap project, 1000 Genome Project, and the healthy Chinese adults database from BGI. After filtering against these databases, four variants that might be associated with RDs diseases were left: The first was a heterozygous nucleotide deletion variation c.942_944delGAA in *IMPDH1,* which was predicted to result in lysine deletion at position 314 (p.Lys314del p.K314del) in the proband of Family-012; the second was a homozygous single-nucleotide-polymorphic site c.1924T>A in *ABCA4* leading to amino acid substitution of phenylalanine for isoleucine at position 642 (p.Phe642Ile p.F642I) in the proband of Family-024; the third and fourth were compound heterozygous mutations of c.272A>G and c.196C>T in *NMNAT1* that caused glutamic acid substitution for glycine at position 91 (p.Glu91Gly p.E91G) and amino acid substitution of arginine for tryptophan at position 66 (p.Arg66Trp p.R66W) in the proband of Family-035. The mutations of *ABCA4* c.1924T>A and *NMNAT1* c.272A>G were novel and were first identified as associated with RDs, while *IMPDH1* c.942_944delGAA and *NMNAT1* c.196C>T have been previously reported to be pathogenic mutations that cause RP and LCA [5,8]. The Sanger sequencing results confirmed these variants (Figure 4), and they cosegregated with respective RDs phenotype in the families (Figure 1). The prediction analysis indicated that these variants were highly conserved across species (Figure 4). In addition, SIFT and PolyPhen2 were performed to predict the effect of the novel missense mutation: *ABCA4* c.1924T>A was possibly damaging according to SIFT, and *NMNAT1* c.272A>G was possibly damaging according to PolyPhen2 (Table 2).

DISCUSSION

We reported four mutations associated with different RDs in three Chinese families: *IMPDH1* c.942_944delGAA with adRP, *ABCA4* c.1924T>A with STGD, and *NMNAT1* c.272A>G and c.196C>T with LCA. To the best of our knowledge, our study is the first to describe the mutations of *ABCA4* c.1924T>A and *NMNAT1* c.272A>G are associated with RDs. Target regions of known RD gene sequencing–based NGS was used to identify these variations, and Sanger sequencing of candidate mutations verified these mutations cosegregated with the disease phenotypes in the respective families, which were absent in the BGI normal controls database. Prediction analysis of evolutionary conservation and human nsSNPs functional effects suggested that this mutation might be highly deleterious.

IMPDH1 encoding inosine monophosphate dehydrogenase 1 widely expressed rate-limiting enzyme of the de novo pathway of guanine nucleotide biosynthesis and was

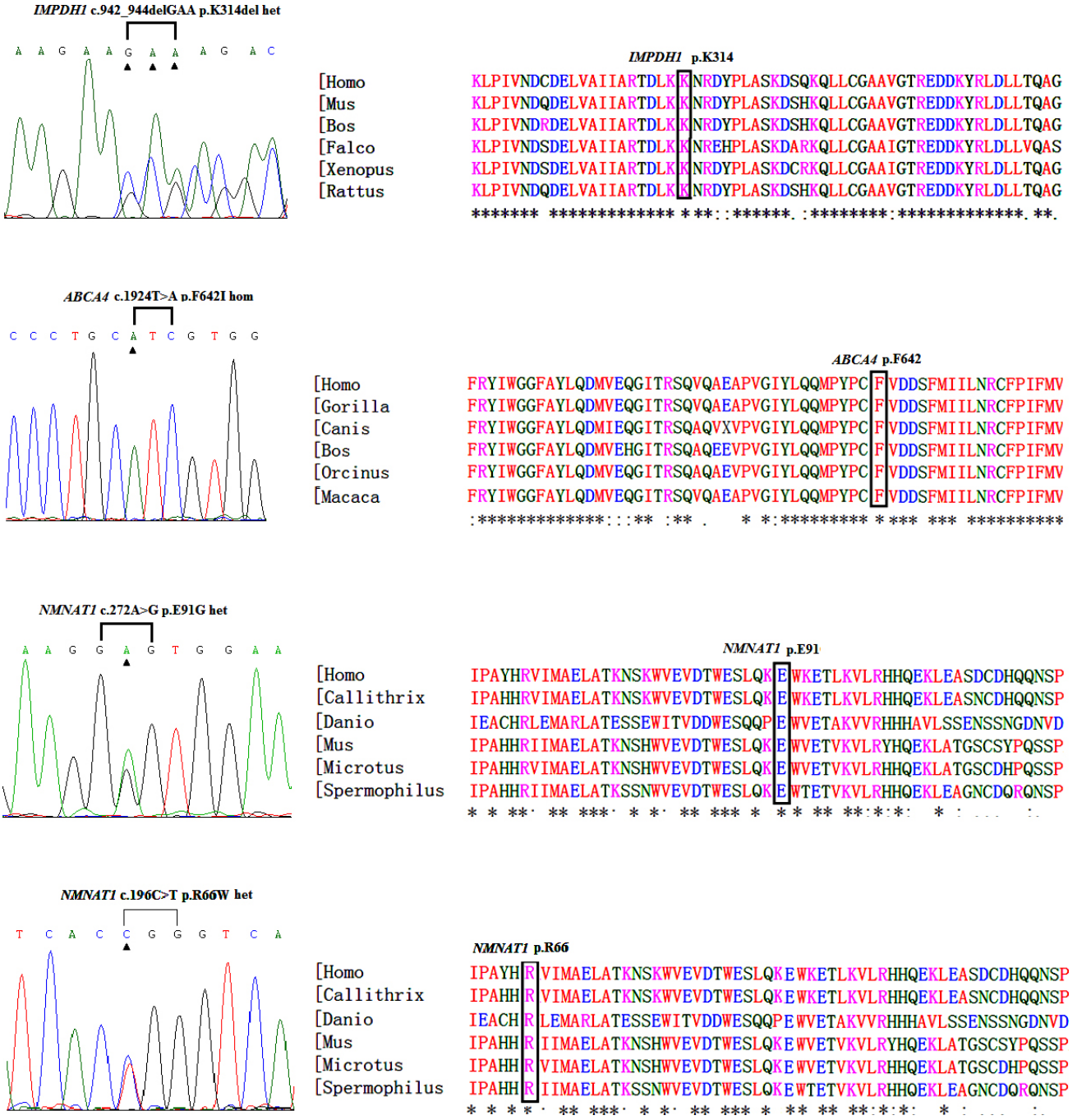

Figure 4. Pathogenic mutations and orthologs. The arrows indicate the mutations in the respective pathogenic genes: heterozygous nucleotides deletion variation c.942_944delGAA in *IMPDH1*, homozygous single-nucleotide-polymorphic site c.1924T>A in *ABCA4*, and compound heterozygous single-nucleotide-polymorphic sites c.272A>G and c.196C>T in *NMNAT1*.The amino acids p.K314, p.F642, p.E91, and p.R66 located in IMPDH1 ABCA4 and NMNAT1 are marked with the column and are highly conserved among all species shown.

Table 2. Summary of mutations in three disease-causing genes.

Sample	Disease	Inheritance manner[a]	Gene	Exon	Nucleotide change	Amino acid change	Mutation type[b]	SIFT/ Polyphen2[c]
Family-012	RP	AD	*IMPDH1*	10	c.942_944delGAA	p.K314del	het	N.P.
Family-024	STD	AR	*ABCA4*	13	c.1924T>A	p.Phe642Ile	hom	0.03/ 0.278
Family-035	LCA	AR	*NMNAT1*	3	c.272A>G	p.Glu91Gly	het	0.2/0.745
			NMNAT1	3	c.196C>T	p.Arg66Trp	het	N.P

[a]Inheritance manner: AR (autosomal recessive), AD (autosomal dominant);[b] Mutation type: het (heterozygous), hom (homozygous); [c]SIFT/ Polyphen2: N.P. (not predicted)

identified in 2002 as associated with RP by linkage mapping [9]. *IMPDH1* might account for 5% to 10% of adRP cases among Americans and Europeans [10,11]. Patients with *IMPDH1* mutations presented severe autosomal dominant degenerative retinopathy, while it was interesting that *Impdh1*$^{(-/-)}$ null mice displayed a slowly progressive form of retinal degeneration [11,12]. Therefore, IMPDH1 misfolding and aggregation, rather than reduced enzyme activity, were the likely cause of the severe phenotype in the human form. *IMPDH1* c.942_944delGAA was first identified as an adRP mutation in a European family in 2013 [5]. All affected members in Family-012 carried this mutation and presented relatively earlier onset of symptoms and similar disease severity compared with American and European patients with *IMPDH1* mutations. We analyzed the RP phenotype associated with *IMPDH1* c.942_944delGAA and confirmed the pathogenicity of the mutation.

The *ABCA4* gene produces a photoreceptor-specific ATP-binding cassette transporter involved in clearance from photoreceptor cells of the byproduct of the retinoid cycle of vision [13]. The spectrum of retinal dystrophies caused by mutations in the *ABCA4* gene is broad, and includes severe RP, STGD, cone-rod dystrophy, age-related macular degeneration, and so on. Diverse phenotypes related to *ABCA4* might perplex the clinical diagnosis sometimes. Fundus manifestations of patients in Family-024 seemed similar to RP, and all patients had complained of night blindness; however, atrophic-appearing RPE concentrating in the region around the macular and delayed and reduced ERG responses were in accordance with STGD Group 3, which had macular and generalized cone and rod system dysfunction [14,15]. Therefore, the diagnosis of Family-024 was corrected for STD, and *ABCA4* c.1924T>A was identified as the causative mutation for this family.

LCA is the most severe human form of inherited photoreceptor-neuron degeneration resulting in congenital blindness, with an incidence of approximately 1 in 80,000 [16]. Seventeen genes have been implicated in the LCA. *NMNAT1* was first mapped to 1p36 through linkage analysis in a large consanguineous Pakistani family with LCA in 2003; however, it was not accurately identified until 2012 [8]. *NMNAT1* encodes the nuclear isoform of nicotinamide mononucleotide adenylyltransferase, a rate-limiting enzyme in nicotinamide adenine dinucleotide (NAD^+) biosynthesis [17,18]. It was suggested that *NMNAT1* mutations could cause severe and rapid macular degeneration, in addition to the typical LCA phenotype of nystagmus, severe loss of vision, and abnormal ERGs. The phenotype associated with *NMNAT1* mutations was classified in LCA9. Patients with LCA9 presented a peculiar, prominent retinal feature termed macular coloboma that consists of an atrophic lesion in the central retina with a pigmented border, and the remainder of the retina was abnormal as well, with pigmentary changes, attenuated retinal blood vessels, and optic disc pallor [19]. The symptoms and fundus manifestations of the two affected individuals in Family-035 were exceedingly compatible with LCA9, including early onset of nystagmus, severe loss of vision, macular coloboma, and so on. The compound heterozygous variants in *NMNAT1*, c.196C>T and c.272A>G, cosegregated with the phenotypes of Family-035. *NMNAT1* c.196C>T has been detected in a Pakistani LCA9 pedigree and was proved to decrease NMNAT1enzyme activity [20]. *NMNAT1* c.272A>G is a novel mutation, which was absent in previous reports of the pathogenic mutations spectrum causing LCA9.

Thus, in total, we have identified four mutants in three Chinese families associated with adRP, STGD, and LCA, including a deletion mutation encoding p.K314del in *IMPDH1* and three missense mutations encoding p.F642I in *ABCA4,* p.E91G, and p.R66W in *NMNAT1* with gene panel–based NGS. All mutations were validated with directed PCR and Sanger sequencing. Identification of these mutations reaffirms the clinical and genetic heterogeneity of RDs, and further expands the mutation spectrum of RDs.

APPENDIX 1. KNOWN CAUSATIVE GENES RESPONSIBLE FOR RDS.

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

ACKNOWLEDGMENTS

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