Identification of Novel *EYS* Mutations by Targeted Sequencing Analysis

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Purpose: Retinitis pigmentosa (RP) is an inherited and progressive degenerative retinal disease that often results in severe vision loss and blindness. However, mutations in known RP disease genes account for only 60% of RP cases, indicating that there are additional pathogenic mutations yet to be identified. We aimed to identify the causative mutations in the eyes shut homolog (*EYS*) gene in a cohort of Chinese RP and rod-cone dystrophy families.

Materials and Methods: Targeted next-generation sequencing was applied to identify novel mutations in these patients. Candidate variants were evaluated using bioinformatics tools. Mutations were confirmed by Sanger sequencing.

Results: We identified eight heterozygous mutations in the *EYS* gene in the four probands, including a novel frameshift deletion mutation, c.8242_8243del (p.L2748fs); a novel insertion mutation, c.5802_5803insT (p.I1935YfsX6); a novel splicing mutation, c.1300-1G>A; two heterozygous stop-gain mutations, c.1750G>T (p.E584X) and c.8805C>A (p.Y2935X); and three novel missense mutations, c.8269G>A (p.V2757I), c.2545C>T (p.R849C) and c.7506C>A (p.S2502R). Only c.8805C>A had been reported previously in RP patients. None of these mutations were present in 1000 control individuals.

Conclusions: We identified seven novel mutations in the *EYS* gene, expanding the mutational spectra of *EYS* in Chinese patients with RP and rod-cone dystrophy.

Keywords: NGS, *EYS* mutation, autosomal-recessive retinitis pigmentosa

Introduction

RETINITIS PIGMENTOSA (RP, OMIM# 268000) refers to progressive degeneration of the retina, which exhibits genetic heterogeneity and a complex clinical phenotype (Daiger *et al.*, 2013). Unfortunately, the pathogenesis of RP has not been completely elucidated (Pagon, 1988; Yang *et al.*, 2020). RP is characterized by degeneration of rod cells, cone cells, and retinal pigment epithelial cells and by progressive functional impairment of the outer retina (Hamel, 2007). Patients with RP first develop night blindness and gradual loss of vision, and the disease progresses to classical fundus manifestations, such as bone spicule-like pigmentation of the retina (Tsujikawa *et al.*, 2008; Khan *et al.*, 2015). RP has a global prevalence of 1 in 3500 to 4000 individuals and affects ~1 million individuals (Hartong *et al.*, 2006; Chen *et al.*, 2014).

RP can be inherited in autosomal-dominant (30–40%), autosomal-recessive (arRP, 50–60%), or X-linked (5–15%) patterns (Allard, 1983; Maria *et al.*, 2015). In addition, the absence of a family history precludes the determination of its mode of inheritance, a condition known as sporadic RP (sRP). RP-related genes encode proteins that participate in visual signal transduction, the rhodopsin cycle, photoreceptor structure, and transcriptional regulation of photoreceptor cells. Mutations in these genes influence multiple biochemical pathways, including RNA intron splicing (Zhou *et al.*, 2002; Maita *et al.*, 2004), structural or cytoskeletal function (Gu *et al.*, 1998; Clarke *et al.*, 2000; Liu *et al.*, 2004), synaptic or cell–cell interaction (Chapple *et al.*, 2003; Reiners *et al.*, 2005), and vitamin A metabolism (Xue *et al.*, 2004; Moiseyev *et al.*, 2005). More than 90 genes have been identified to be associated with

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RP and account for ~60% of RP cases (RetNet website) (Zhang *et al.*, 2018).

Eyes shut homolog (*EYS*, OMIM# 612424) is one of the largest genes expressed in the retina. *EYS* was first identified as the gene causing arRP, located at the RP25 locus (6q12) and spanning a genomic region of more than 2 Mb on chromosome 6 (Abd El-Aziz *et al.*, 2008). The human *EYS* gene encodes an extracellular protein of 3165 amino acid residues, which starts with a signal peptide and contains 28 epidermal growth factor (EGF)-like domains followed by 5 LamG domains (Collin *et al.*, 2008). *EYS* was reported to interact with matriglycan of *O*-mannosyl glycans by its LamG domains (Liu *et al.*, 2020). The *EYS* protein is proposed to play a role in the modeling of retinal architecture in the human eye and required for ommatidium development in *Drosophila* (Husain *et al.*, 2006; Zellhof *et al.*, 2006). In zebrafish, *EYS* protein is localized to the connecting cilium/transition zone of photoreceptors and ablation of *eyes* in zebrafish leads to mislocalization of outer segment proteins, F-actin disruption, and cone-rod dystrophy (Yu *et al.*, 2016). Mutations in *EYS* have also been identified in cone-rod dystrophy patients (Littink *et al.*, 2010; Katagiri *et al.*, 2014; Sengillo *et al.*, 2018; Pierrache *et al.*, 2019) in addition to patients with RP. *EYS*-associated retinal dystrophy is a common inheritable retinal dystrophy (IRD) in European and Japanese populations (Abd El-Aziz *et al.*, 2010; Audo *et al.*, 2010; Barragán *et al.*, 2010; Arai *et al.*, 2015; Chen *et al.*, 2015; Maeda *et al.*, 2018). *EYS* mutations account for 15.9% of arRP cases in a Spanish population (Barragán *et al.*, 2010). A high prevalence (18–23%) of *EYS*-associated RP was reported in Japanese RP patients (Arai *et al.*, 2015; Yang *et al.*, 2020). In Chinese RP patients, the p.C2139Y mutation affecting the EGF-like domain of the *EYS* protein is a hotspot mutation (Gu *et al.*, 2016; Xiao *et al.*, 2019). However, the relationships between *EYS* variants and accurate clinical phenotypes are still uncertain because of the lack of large cohort studies in Chinese populations.

Next-generation sequencing (NGS), a recently emerging technique, has the advantages of large-scale, rapid, and systematic identification of variants. NGS approaches generally include targeted capture array sequencing,

whole-exome sequencing, and whole-genome sequencing. Compared with traditional approaches for screening potential genes such as positional cloning and gene identification, NGS provides a novel and efficient technique for molecular diagnosis of RP.

In this study, we utilized a targeted NGS approach to assess causative mutations underlying the cases in a cohort of arRP and rod-cone dystrophy families from central and southwest China and identified eight compound heterozygous mutations in the *EYS* gene: c.8242_8243del (p.L2748fs), c.8269G>A (p.V2757I), c.2545C>T (p.R849C), c.5802_5803insT (p.I1935YfsX6), c.7506C>A (p.S2502R), c.1750G>T (p.E584X), c.8805C>A (p.Y2935X), and c.1300-1G>A. Except for c.8805C>A (p.Y2935X), all these mutations were novel mutations not reported previously. Our findings expanded the *EYS* mutation spectrum in Chinese patients with arRP and rod-cone dystrophy.

Materials and Methods

Patient recruitment and ethics statement

This study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Ethics Review Boards of Sichuan Provincial People's Hospital and Henan Eye Institute. Written informed consent was obtained from all patients and their family members. All experiments were performed following the approved protocols.

Genetic analysis by targeted NGS

Genomic DNA was extracted from peripheral blood samples obtained from RP patients, unaffected relatives, and control individuals with DNA extraction kits (TianGen, Beijing, China) according to the manufacturer's instructions. DNA samples were subjected to targeted NGS analysis according to the previously described panel (Wang *et al.*, 2013). In brief, DNA extracts were sheared into 250–500 bp segments, and the sheared fragments were end-repaired with polynucleotide kinase. An A base was added to the 3' ends with Klenow exonuclease. Finally, Illumina Y-shaped index

TABLE 1. PRIMERS AND POLYMERASE CHAIN REACTION CONDITIONS USED IN THIS STUDY

Amplicon	Sequence (5' to 3')	Annealing temperature (°C)	Products (bp)
c.1300-1G>A	F-AGCCCATCAAACAGAAGATTAG R-CTCCACAGTAGCAGAGGTGT	58	362
c.1750G>T	F-TGGATGCAGTTGTTTGAGTGA R-TCTTCCCTCCTTTTATTGTGCT	58	372
c.2545C>T	F-TGGATGGACTGGACAGAACT R-GTCCCCTACCCACAATGTACA	62	374
c.5802-5803insT	F-AAGAGGCAGGAAAGAGACGT R-AATACTTGGTTGTTGTTGGTTC	60	495
c.7506C>A	F-TGGGGCTGGGAATAACAACA R-GGCATCATGGGCTATTACCC	60	438
c.8242_8243del	F-TGAGAACTGTCCACAACCTAGAG R-CCTTCTGCACCAACTCTTCC	62	386
c.8269G>A	F-TGAGAACTGTCCACAACCTAGAG R-CCTTCTGCACCAACTCTTCC	62	386
c.8805C>A	F-AGGTGGCTCAAATGTAGGTGA R-TGGAGACCAATTGCCAGAAA	61	444

FIG. 1. Clinical data of the proband of family HEI-133. (A) Fundus photograph showing pigment deposits and retinal atrophy. (B) The visual field test suggested the loss of peripheral vision in both eyes. (C) Scotopic ERG indicated reduced amplitudes of both the a- and b-waves. (D) Photopic ERG indicated reduced amplitudes of both the a- and b-waves. (E) Reduced response in the flicker ERG test. ERG, electroretinogram.

FIG. 2. Clinical data of the proband of family SCRP-18. **(A)** Fundus photograph showing deficiency of pigmented epithelial tissue with narrowed arterioles and pigment deposition. The *arrows* indicate macular regions. **(B)** OCT revealed disorganized photoreceptor layers. **(C)** Autofluorescence imaging of both eyes showed slightly increased fluorescence. The macular region exhibited small diffuse regions of high fluorescence, and the vascular arch exhibited pigment proliferation and fluorescence shadowing. **(D)** The visual field test suggested the loss of both peripheral and central vision in both eyes. OCT, optical coherence tomography.

adapters were used to construct paired-end DNA libraries, and the captured libraries were sequenced in an Illumina HiSeq 2000 sequencer (Illumina, Inc., San Diego, CA).

Sequencing reads were aligned to the reference human genome assembly hg38 UCSC using the Burrows-Wheeler Aligner (BWA) program. The “mpileup” command in SAMTOOLS v.0.1.19 was applied to identify insertions/deletions and single nucleotide variants. To filter and annotate the detected variants, three databases were referenced: NCBI CCDS, RefSeq, and Ensembl. The following exclusion steps were applied: Variants within intergenic, intronic, and untranslated regions and synonymous mutations were excluded. In addition, benign variants were eliminated with the online prediction tools PolyPhen-2 and SIFT. Mutations identified in our study were verified in the HGMD database to confirm whether the pathogenic mutations had been previously reported.

Mutation verification by Sanger sequencing

Sanger sequencing was carried out to confirm the potential mutations in the RP families. Polymerase chain reaction (PCR) primers (Table 1) were designed and synthesized by

Sangon Biotech (Shanghai, China) to amplify DNA fragments flanking the mutation site. PCR products were purified with FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific Fermentas, Waltham, MA) and were then sequenced in an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA).

Results

Clinical assessments

Targeted NGS analysis was performed on two Chinese arRP families and one rod-cone dystrophy family from the central and southwest regions of China. The proband of family HEI-133 was a 61-year-old man who exhibited night blindness, peripheral vision loss, and a reduced electroretinogram response (Fig. 1). The proband of family SCRP-18 III:1 was a 32-year-old man who experienced night blindness, progressive peripheral vision loss, optic disc atrophy, and retinal vascular stenosis (Fig. 2). Fundus images showed slight pigment deposition (Fig. 2A). Optical coherence tomography revealed disorganized photoreceptor and ellipsoid layers (Fig. 2B). Autofluorescence imaging of both eyes showed hypofluorescence in the macular region, a

FIG. 3. Pedigree of the HEI-133 family and corresponding sequencing chromatograms. (**A–E**) Pedigree chart and *EYS* mutations identified in the HEI-133 family. The proband, II-6, carries the compound heterozygous mutations c.8243_8244del (M1) and c.8269G>A (M2). M1/M2 indicates individuals with compound heterozygous mutations; M1/+ indicates a heterozygous carrier; and +/+ indicates an individual carrying two wild-type alleles. Arrow indicates the affected proband. DEL AA indicates deletion of the AA dinucleotide; REF indicates the reference sequence. *EYS*, eyes shut homolog.

hyperfluorescent ring in the posterior polar region, and diffuse hypofluorescent lesions beyond the vascular arch (Fig. 2C). The visual field test suggested the loss of peripheral and central vision in both eyes (Fig. 2D). This patient was classified as having rod-cone dystrophy. His brother III:4 was also affected.

Analysis of causative mutations

The frameshift deletion mutation c.8242_8243del (p.L2748fs, M1) and the heterozygous mutation c.8269G>A (p.V2757I, M2) in the *EYS* gene were identified in the proband II:6 of family HEI-133 (Fig. 3A). Deletion of -a dinucleotide resulted in a frameshift mutation and a truncated protein. Moreover, the heterozygous mutation c.2545C>T

(p.R849C, M3) and the heterozygous insertion frameshift mutation c.5802_5803insT (p.I1935YfsX6, M4) were detected in the proband III:1 of family SCR-18 (Fig. 4A). To validate these two mutations, we next collected DNA samples from the proband's parents for Sanger sequencing. The proband's father carried the c.2545C>T (p.R849C) mutation, whereas his mother carried the c.5802_5803insT (p.I1935YfsX6) mutation. His affected brother III:3 also carried the compound heterozygous mutations M3/M4. Unaffected brother III:2 carried heterozygous M4 allele and unaffected sister III:3 carried heterozygous M3 allele. Insertion of a T nucleotide led to a reading frameshift mutation and a truncated protein. In family arRP-30 (Fig. 4B), the heterozygous missense mutation c.7506C>A (p.S2502R, M5) and the heterozygous stop-gain mutation c.1750G>T

FIG. 4. Pedigrees of the SCR-P-18 and arRP-30 families and the corresponding sequencing chromatograms. **(A)** Pedigree chart and *EYS* mutation identified in the SCR-P-18 family. The proband, III-1, carries the compound heterozygous mutations c.2545C>T (M3) and c.5802-5803insT (M4). M3/M4 indicates individuals with compound heterozygous mutations. His father II:1 carries M3/+ allele and his mother carries +/M4 allele. Arrow indicates the affected proband. His affected brother III:3 also carried the compound heterozygous mutations M3/M4. Unaffected brother III:2 carries heterozygous M4 allele and unaffected sister III:3 carries heterozygous M3 allele. **(B)** Pedigree chart and *EYS* mutations identified in the family arRP-30. The proband, II-1 carries the compound heterozygous mutations c.7506C>A (M5) and c.1750G>T (M6). M3/M4 indicates individuals carrying both mutations as compound heterozygous mutations. His father II:1 carried M5/+ allele and his mother carried +/M6 allele. M5/+ and +/M6 indicate heterozygous carriers. His affected sister II:2 also carried the compound heterozygous mutations M5/M6. Unaffected brother II:3 carried heterozygous M6 allele. Arrow indicates the affected proband. **(C)** The C.8805C>A (M7) mutation and c.1300-1G>A (M8) mutations in *EYS* were identified in a patient with sporadic arRP-35. Arrows indicate the altered nucleotides.

(p.E584X, M6) were identified in *EYS*. Sanger sequencing analysis confirmed that the proband's father carried the c.7506C>A (p.S2502R, M5) mutation and that his mother carried the c.1750G>T (p.E584X, M6) mutation. His affected sister II:2 also carried the compound heterozygous mutations M5/M6. Unaffected brother II:3 carried heterozygous M6 allele. The c.1750G>T (p.E584X, M6) mutation is a nonsense mutation that results in premature termination of *EYS* protein translation (Fig. 5A).

In addition, in 12 patients with sRP in family arRP-35, we found 2 compound heterozygous mutations in the *EYS* gene (Fig. 4C). Specifically, the known nonsense mutation c.8805C>A (p.Y2935X, M7) and the novel splicing mutation c.1300-1G>A (M8) in *EYS* were identified. The splicing mutation c.1300-1G>A (M8) led to the production of a truncated protein (Fig. 5A).

These 7 novel heterozygous mutations had not been previously reported in patients with RP and rod-cone dystrophy and

FIG. 5. *EYS* mutations in protein domains and sequence alignment of the affected amino acid residues. **(A)** Distribution of different mutations across the various domains of the *EYS* protein. **(B)** Comparison of the *EYS* amino acid sequence across homologous genes in various species. *C. lupus*, *Canis lupus*; *D. rerio*, *Danio rerio*; *F. catus*, *Felis catus*; *H. sapiens*, *Homo sapiens*; *P. abelii*, *Pan abelii*; *V. vulpes*, *Vulpes vulpes*.

were not present in our in-house database of 1000 ethnicity-matched control samples. Those seven amino acid residues affected by these seven mutations identified in this study are highly conserved in vertebrates, indicating the importance of these residues (Fig. 5B). In addition, the effects of these mutations were predicted with the online tools PolyPhen-2 or SIFT, and the frequencies of these mutations were analyzed in the Genome Aggregation Database (gnomAD) (Table 2). Ac-

cording to the American College of Medical Genetics and Genomics (ACMG) guidelines, the three frameshift mutations (p.L2748fs, p.I1935YfsX6, and p.E584X) and one splicing mutation (c.1300-1G>A) identified in this study are likely causative mutations. The three missense mutations (p.V2757I, p.R849C and p.S2502R) should be classified as variants of uncertain significance. In summary, our data expand the mutation spectrum of *EYS* in the Chinese population.

TABLE 2. SUMMARY OF PATHOGENIC VARIANTS IN *EYS*

Pedigree	Nucleotide mutations	Amino acid changes	Frequencies in gnomAD	SIFT (cutoff=0.05)	PolyPhen-2 (cutoff=1)	DbSNP-IDs
HEI-133	c.8242_8243del	p.L2748fs	0.000006722	N/A	N/A	rs1240944758
	c.8269G>A	p.V2757I	0.000006381	Deleterious (0.04)	Deleterious (0.536)	rs775398034
SCR-18	c.2545C>T	p.R849C	0.00005858	Deleterious (0.03)	Deleterious (0.997)	rs369347845
	c.5802-5803insT	p.I1935YfsX6	0.000006997	Deleterious (0.01)	Deleterious (0.969)	rs1455826633
arRP-30	c.1750G>T	p.E584X	0.000004003	N/A	N/A	rs527236072
	c.7506C>A	p.S2502R	0.0001648	Neutral (0.07)	Deleterious (1.000)	rs77113519
arRP-35	c.8805C>A	p.Y2935X	0.00003719	N/A	N/A	N/A
	c.1300-1G>A	N/A	0.000003988	N/A	N/A	N/A

DbSNP, the single nucleotide polymorphism database; *EYS*, eyes shut homolog; N/A, not available.

Discussion

Mutations in the *EYS* gene are recognized as a significant cause of arRP. *EYS* mutations were found to account for 15.9% of arRP cases in a Spanish population and 23% of arRP cases in Japanese patients (Pagon, 1988; Barragán *et al.*, 2010). In this study, we used targeted NGS to analyze a cohort of Chinese arRP and rod-cone dystrophy families and identified eight heterozygous mutations in the *EYS* gene: c.8242_8243del, c.8269G>A, c.2545C>T, c.5802_5803insT, c.7506C>A, c.1750G>T, c.8805C>A, and c.1300-1G>A (Fig. 5). The proband of family SCRP-18 exhibited rod-cone dystrophy symptoms and carried c.2545C>T and c.5802_5803insT. Similarly, Yang *et al.* (2020) reported rod-cone dystrophy patients carrying *EYS* mutations in a Japanese population (Pagon, 1988). Therefore, different mutations in *EYS* could cause distinct phenotypes in patients.

In this study, the identified c.5802_5803insT (p.I1935YfsX6) mutation resulted in a frameshift change at codon 1935 and a truncated protein, disrupting the Laminin G-like domain. The stop-gain mutation c.1750G>T (p.E584X) disrupted translation at codon 584 and produced a truncated protein that lacked most of the calcium-binding EGF-like domain. The stop-gain mutation c.8805C>A (p.Y2935X), the missense mutation c.8269G>A (p.V275I), and the frameshift deletion mutation c.8242_8243del (p.L2748fs) were located in the same exon and EGF domain. The stop-gain mutation c.8805C>A (p.Y2935X) was located at codon 2935, resulting in a truncated protein. The c.2545C>T (p.R849C) and c.7506C>A (p.S2502R) mutations were located at codon 849 and codon 2502, respectively, and affected conserved amino acid residues (Fig. 5). Therefore, these identified mutations might be pathogenic mutations and could contribute to the development of photoreceptor degeneration.

In summary, our study identified five novel biallelic heterozygous *EYS* mutations in RP patients by a targeted NGS approach. In addition, two biallelic mutations in a rod-cone dystrophy patient were identified. Our study expands the mutation spectrum of the *EYS* gene in the Chinese population.

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No competing financial interests exist.

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