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Novel mutations in *CYP4V2* in Bietti corneoretinal crystalline dystrophy: Next-generation sequencing technology and genotype-phenotype correlations

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Purpose: To identify any novel mutations in *CYP4V2* in 85 Chinese families with Bietti corneoretinal crystalline dystrophy (BCD) by using next-generation sequencing, and to summarize the mutation spectrum in this population, along with any genotype-phenotype correlations.
Methods: A total of 90 patients with BCD from 85 unrelated Chinese families were recruited. All probands were analyzed by using gene chip-based next-generation sequencing, to capture and sequence all the exons of 57 known hereditary retinal degeneration-associated genes. The candidate variants were validated with PCR and Sanger sequencing.
Results: Twenty-eight mutations were detected in all patients, including thirteen novel mutations (five missense, six deletions, one splicing and one frame-shift mutations) and 15 previously reported mutations. Mutations in 64 patients were inherited from their parents, while three patients had de novo mutations. c.802–8_810del17insGC was the most common mutation, accounting for 78% of the mutations. Although 16 patients were homozygous at this site, the clinical features of all 16 patients were highly heterogeneous.
Conclusions: These results expand the spectrum of mutations in *CYP4V2*, and suggest that mutations in *CYP4V2* may be common in the Chinese population. The phenotype of patients with the homozygous mutation (hom.c.802–8_810del17insGC) is highly heterogeneous.

Bietti corneoretinal crystalline dystrophy (BCD, OMIM 210370) is an autosomal recessive retinal dystrophy, characterized by numerous tiny glistening yellow-white crystals scattered at the posterior pole of the retina, progressive atrophy of the RPE, and choroidal sclerosis. The majority of the cases also present similar crystals at the corneoscleral limbus, and within the vitreous cavity. The disease usually occurs in the second or third decade of life, and progresses to legal blindness by the fifth or sixth decade [1]. *CYP4V2* (Gene ID 285440,OMIM 210370) is currently the only identified gene associated with this disease [2,3]. BCD is relatively common in Chinese and Japanese populations [4-25], who harbor the c.802–8_810del17insGC in exon 7, c.992A>C in exon 8, and c.1091–2A>G in exon 9 mutations. These most common mutations account for 83.3% of the mutant alleles.

In a previous report, we described the spectrum of mutations in *CYP4V2* in 92 Chinese patients with BCD, with Sanger sequencing [18]. Compared to Sanger sequencing, next-generation sequencing (NGS) targets the genes known to underlie BCD, and thus, has been used to encompass clinical and genetic heterogeneity of this disease group. Patients with BCD were commonly reported in an East Asian population with an estimated gene frequency of 0.005 in China [26]. The incidence of BCD also appears in a large proportion of Chinese patients with retinitis pigmentosa. To understand the spectrum of mutations in *CYP4V2* within the Chinese population, further genetic counseling is essential for the diagnosis and treatment of the disease. The present study assessed the distribution and prevalence of mutations and genes involved in BCD among the Chinese population, using a comprehensive analysis of BCD through the application of an NGS panel comprising 57 genes optimized from a previous report [27].

In this study, we detected mutations in the *CYP4V2* gene in 85 BCD probands from southwestern China, summarized the mutation spectrum, and analyzed the genotype-phenotype correlations.

METHODS

Recruitment of subjects: Ninety patients from 85 Chinese families with BCD were recruited from the Southwest Eye Hospital and Southwest Hospital, Chongqing, China. There were no systemic diseases such as hypertension, examinations. Family members of the probands were also

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invited for clinical examination and genetic assessment. The research protocol was approved by the Ethics Review Board of the Southwest Hospital (Chongqing, China), and the study was performed in accordance with the Declaration of Helsinki and adhered to the ARVO statement on human subjects. Written informed consent was obtained from all participants. All patients underwent complete eye examinations, including best-corrected visual acuity (BCVA) testing with the fractional VA chart, slit-lamp biomicroscopy, fundoscopy, fundus photodocumentation, fundus autofluorescence (AF), fundus fluorescein angiography (FFA), spectral domain optical coherence tomography (SD-OCT), and a full-field electroretinogram (FERG).

Targeted region capture and next-generation sequencing: For exome sequencing, we fragmented 1–3 µg of genomic DNA, extracted from each sample, to an average size of 180 bp with a Bioruptorsonicator (Diagenode, Liege, NJ). Paired-end sequencing libraries then were prepared using the DNA Sample Prep Reagent Set 1 (NEBNext, San Diego, CA). Library preparation included end repair, adaptor ligation, and PCR enrichment, and was performed as recommended by Illumina protocols.

Next, 57 genes associated with ophthalmologically diseases (Appendix 1) were selected with a gene capture strategy, using the GenCap custom enrichment kit (MyGenostics Inc., Beijing, China), following the manufacturer's protocol. The biotinylated capture probes (80–120-mer), were designed to tile all of the exons with non-repeated regions. Briefly, the 1 µg DNA library was mixed with Buffer BL and the GenCap gene panel probe (MyGenostics), and heated at 95 °C for 7 min and 65 °C for 2 min on a PCR machine. Then, 23 µl of the 65 °C prewarmed Buffer HY (MyGenostics) was added to the mix, and the mixture was held at 65 °C with PCR lid heat on for 22 h for hybridization. Then, 50 µl MyOne beads (Life Technology, Carlsbad, CA) was washed in 500 µl 1X binding buffer three times, and resuspended in 80 µl 1X binding buffer. About 64 µl 2X binding buffer was added to the hybrid mix, and transferred to the tube with the 80 µl MyOne beads. The mix was rotated for 1 h on a rotator. The beads were then washed with WB1 buffer at room temperature for 15 min once and WB3 buffer at 65 °C for 15 min three times. The bound DNA was then eluted with buffer elute. The eluted DNA was finally amplified for 15 cycles using the following program: 98 °C for 30 s (1 cycle); 98 °C for 25 s, 65 °C for 30 s, 72 °C for 30 s (15 cycles); and 72 °C for 5 min (1 cycle). The PCR product was purified using SPRI beads (Beckman Coulter, Brea, CA) according to the manufacturer's protocol. The enrichment libraries were sequenced on the Illumina HiSeq X ten sequencer for a 150 bp paired read.

Variant identification and validation: After sequencing, the raw data were saved in the FASTQ format. Then, the bioinformatics analysis was performed: First, Illumina sequencing adapters and low-quality reads (<80 bp) were filtered by cutadapt. After quality control, the clean reads were mapped to the UCSC hg19 human reference genome using BWA [27] (http://bio-bwa.sourceforge.net/). Duplicated reads were removed using picard tools, and mapping reads were used to detect variations. Second, single nucleotide polymorphism (SNPs) and insertion and deletion variants were detected with GATK HaplotypeCaller. Then, using GATK VariantFiltration to filter the variants, the filtered standard as follows: a) variants with mapping qualities <30; b) the total mapping quality zero reads <4; c) approximate read depth <5; d) QUAL<50.0; and e) phred-scaled p value using Fisher's exact test to detect strand bias >10.0. After the two steps, the data were transformed into the VCF format. Variants were further annotated with ANNOVAR [28], and associated with multiple databases, such as 1000 genome, ESP6500, dbSNP, EXAC, and HGMD, and predicted with Sorting Intolerant From Tolerant (SIFT), PolyPhen-2, MutationTaster, and GERP++. Genomic DNA from all available family members were obtained for Sanger sequencing. The PCR samples were visualized on agarose gels, purified, and sequenced on an ABI PRISM 3730 genetic analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA), using the terminator cycle sequencing method. Sites of variation were identified through a comparison of DNA sequences with the corresponding GenBank reference sequences.

Identification of mutations in CYP4V2: Approximately 221,340 kb of the exons and adjacent intronic regions of 57 genes associated with ophthalmological diseases were captured and sequenced from every proband. The mean coverage of the 57 genes associated with retinitis pigmentosa (RP) was about 99.6%, and the median depth was 509X. A mean of 94.7% of the base pairs with N20X coverage were detected successfully for each gene, indicating a high capability for identifying variants.

The *CYP4V2* sequence was compared with the NCBI reference sequence for the *CYP4V2* transcript variant (GenBank ID; NM_207352.4) for allowing direct comparison with known mutations in *CYP4V2* in the Human Gene Mutation Database (HGMD). To confirm the variants in *CYP4V2*, and to assess the inheritance pattern of Bietti crystalline corneoretinal dystrophy in the probands, Sanger sequencing was used to analyze the variants in the probands and their family members.

RESULTS

Clinical findings: The cohort included five probands whose parents were consanguineous. Of the 85 probands, 46 were male, and 39 were female. The most common initial complaint was poor vision or night blindness starting between the age of 10 and 40 years, followed by vision reduction; three patients complained of visual distortion. Slit-lamp examination revealed crystalline deposits at the corneoscleral limbus in 80 patients. In most patients, fundus examination showed the presence of numerous tiny, glistening yellow-white crystals scattered at the macular area and posterior pole of the retina, atrophy of the RPE, and choroidal sclerosis. VA varied markedly from light perception to 1.0. Only one proband showed a normal visual field, and two patients had normal FERG recordings, while most patients exhibited a reduced FERG.

Concomitant ocular diseases included macular edema in three patients, cataract in 25 patients, choroidal neovascularization (CNV) in one patient, macular hole in two patients, and high myopia in 16 patients. The macular membrane was noted in five patients. Three patients did not show any clinical symptoms in pedigree screening, and none of the patients had hypertension, high blood lipids, or diabetes.

Mutation analysis: According to the complete sequence analysis of the coding and adjacent intronic regions of *CYP4V2*, homozygous, compound heterozygous, and heterozygous *CYP4V2* was detected in 29 families, 50 families, and one family, respectively. A total of 28 mutations were identified, of which seven were novel (Table 1). The mutations c.802–8_810del17insGC, c.1091–2A>G, and c.992 A>G were detected in 47, 22, and 25 unrelated families, respectively. The proportion of these three common mutations was 81.25%. The most common mutation, c.802–8_810del17insGC, was identified homozygous in 16 unrelated families. The homozygous mutation c.992A>C occurred in eight unrelated families, while the homozygous mutation c.1091–2A>G was found in five unrelated families. The compound heterozygous mutations c.802–8_810del17insGC and c.992A>C were identified in seven unrelated families, while c.802–8_810del17insGC and c.1091–2A>G were identified in 15 unrelated families. No statistically significant differences were detected in the age of onset and course of the illness among these types of mutations (Table 2).

Clinical features of patients with a Hom.802–8_810del17insGC mutation: Sixteen probands with BCD (six men and ten women, average age 38 years) harbored a Hom.c.802–8_810del17insGC mutation. The age of onset of vision reduction and night blindness ranged from 10 to 46 years, while the age when patients first visited a doctor ranged from 20 to 68 years (average age, 27.5 years; Appendix 2). We analyzed the clinical features of seven patients with BCD aged between 20 and 30 years [patient 1 (P1), P3, P4, P6, P7, P13, and P15] with an age of onset of 15 to 30 years old. The visual field defects among the seven patients indicated several differences (Appendix 2).

The fundus of the P1, P3, P4, P6, P13, and P15 patients showed signs of severe RPE atrophy, choroid sclerosis, and a rare distribution of crystallization (Figure 1A, P4). Fundus fluorescein angiography of patient P4 showed atrophy of the RPE and choroidal capillaries with mottled hypofluorescence (Figure 1B, P4). VA was decreased markedly with an irregular visual field defect (Figure 1C, P4). Loss of the connector and ellipsoid band of the outer retina, thinning of the RPE, and choroid thickening were shown on the SD-OCT. In addition, severely reduced amplitudes were observed in rod and cone ERGs (Figure 2, P4). Patient P6 exhibited a large amount of crystalline particle deposition in the fundus, slight RPE atrophy (Figure 1F, P6), and slightly reduced amplitudes in rod and cone ERGs (Figure 2, P6). Patient P7 did not show any symptoms of night blindness, and received intravitreal injections of ranibizumab, owing to CNV in the left eye. Only a slight crystalline formation was observed within the macular region of patient P7 (Figure 1 K, P7), although spiced-salt-shaped and map-shaped hypoautofluorescent regions were observed (Figure 1L, P7). VA of the two eyes had decreased slightly (Figure 1H,M), while SD-OCT showed that the structure of the outer retina was complete albeit with choroidal thinning (Figure 1I,J,N,O); however, the ERGs were normal (Figure 2, P7). Patient P9 first presented at 50 years of age, although the onset of symptoms had occurred 10 years earlier, and had been associated with a diffused decrease in VA. Fundus examination showed RPE atrophy, a small amount of crystallization, and slight choroid sclerosis that was limited to the posterior pole. Diffuse and hypoautofluorescence were shown in the AF; however, the FERG was normal. Consequently, a total of 28 mutations were identified, and seven mutations were novel (Table 1). The mutations c.802–8_810del17insGC, c.1091–2A>G, and c.992 A>G were detected in 47, 22, and 25 unrelated families, respectively. The proportion of these three common mutations was 81.25%.

DISCUSSION

We applied targeted NGS technology to detect pathogenic mutations in 85 probands with BCD. Seven novel mutations were found, which expanded the spectrum of mutations in *CYP4V2*. To date, the study group has revealed the spectrum

of mutations in *CYP4V2* of patients with BCD from the Chinese population with the largest sample (including the 92 cases in the previous report) [16]. The most common mutation in *CYP4V2* in the Chinese BCD population is c.802–8_810del17insGC, compared to non-Asian patients with BCD, which indicates potential relationships with coancestors. Thus, mutations in *CYP4V2* are known to be related to ethnic origins.

In a previous study, Sanger sequencing was performed for 92 cases, while 85 cases were subjected to NGS in the present study, indicating that patients carrying a heterozygous *CYP4V2* mutation is a common characteristic of the Chinese population. Only five children from consanguineous marriages and 16 probands from non-consanguineous marriages carried the homozygous c.802–8_810del17insGC mutation. Therefore, we speculated that this deletion/insertion mutation is caused by coancestry by prolonged harboring, and is the genetic basis of the high incidence of BCD in the Chinese population. In some cases, an autosomal recessive inheritance mode was ignored, due to the lack of verification of the parents and the sporadic nature of the BCD patient diagnosis. Therefore, in consideration of the common heterozygous carriers of mutations in *CYP4V2* in the Chinese population, it is necessary to undergo genetic screening and counseling before a birth plan.

The *CYP4V2* gene contains 11 exons spanning 19 kb. The coding sequence begins in exon 1, and continues through exon 11 [29]. *CYP4V2* is homologous to other members of

Table 1. Mutation screening results of the *CYP4V2* of the BCD patients and function analysis of novel mutations in this study.

Exon	Nucleotide change	Amino acid change	report
Exon1	c.65T>A	p.L22H	reported [23]
Intron1	c.215-2A>G	splicing	Reported [15]
Exon2	c.219T> A	p.F73L	Reported [42]
Intron1	c.215-1G>T	splicing	Reported [15]
Exon2	c.283G>A	p.G95R	Reported [7]
Exon4	c.492 delT	p.D164EfsX3	novel
Exon7	c.802-6_810delATACAGGTCATCGCT	p.268_270del	novel
Intron3	c.413+2T>G	splicing	Reported [18]
Exon4	c.414_450del	p.S138Rfs*1	novel
Exon7	c.802-8_810del17insGC	p.V268fs*7	Reported [8]
Exon7	c.810del T	p.A270fs	Reported [26]
Exon7	c.804-6_810del	p.V268_270del	novel
Exon7	c.796_810del	p.268_270del	novel
Exon7	c.802-8-807delCATACAGGTCATC	deleting	novel
Exon7	c.958C > T	p.R320X	Reported [15]
Exon8	c.988G >A	p.G330 R	novel
Exon8	c.992A >C	p.H331P	Reported [3]
Exon8	c.1020G >A	p.W340X	Reported [8]
Exon8	c.1057dupA	p.V355S fs*4	novel
Intron8	c.1091-2A >G	splicing	Reported [8]
Exon9	c.1199G >A	p.R400H	Reported [7]
Exon9	c.1168C >T	p. R390C	Reported [16]
Exon9	c.1187C >G	p.P396R	novel
Exon10	c.1247G>T	p.G416V	novel
Exon10	c.1354C>T	p.R452C	novel
Exon10	c.1384G>C	p.A462P	novel
Exon10	c.1396A >G	p.N466 D	Reported [39]
Intron10	c.1406-1G>A	splicing	novel

Table 2. Clinical features of common *CYP4V2* mutation types in 85 probands with Bietti's Crystalline Dystrophy.

Types	Number of patients	Visual acuity (mean)	Age at consultation (years)	Age at onset (years)	Course of the disease (years)
Hom.c.802-8_810del17insGC	16	0.42±0.54	38.1±12.8	27.0±10.0	10.75±6.07
c.802-8_810del17insGC & c.992A>C	7	0.27±0.23	42.2±7.62	32.6±9.45	9.60±3.26
c.802-8_810del17insGC & c.1091-2A>G	15	0.32±0.24	40.53±10.99	30.47±11.73	10.0±8.95
Hom . c.992A>C	8	0.25±0.25	45.0±7.75	32.12±6.10	12.88±5.99
Hom. c.1091-2A>G	5	0.22±0.12	46.6±6.43	29.6±10.52	17.0±14.07

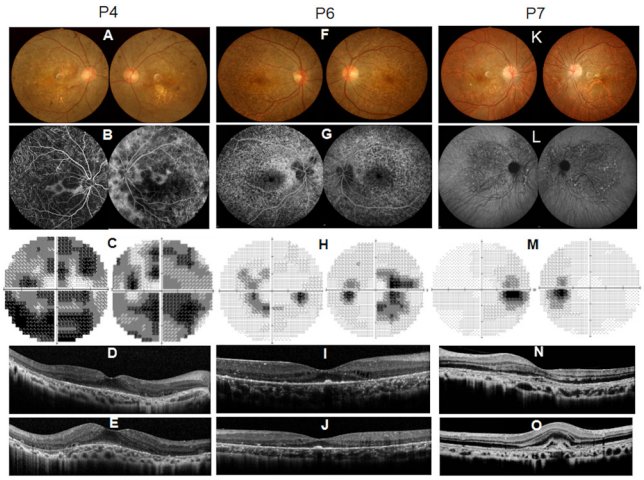

Figure 1. Multimodal imaging testing of three patients with the Hom.c.802–8_810del17insGC mutation. **Patient 4**: Color fundus images (**A**) show normal vasculature, RPE atrophy, choroidal sclerosis, and a rare distribution of crystallization. Fundus fluorescein angiography (FFA) (**B**) shows atrophy of RPE and choroidal capillaries with a mottled hypofluorescence. Visual field (**C**) analysis indicates decreased visual acuity (VA) and irregular visual field defects. Spectral domain optical coherence tomography (SD-OCT) (**D** and **E**) shows the disappearance of the inner segments/outer segments (IS/OS) band, ellipsoid zone (EZ), outer nuclear layer, and outer limiting membrane, and thinning of the thickness in the RPE, retina, and choroid. **Patient 6:** Color fundus images (**F**) show diffused glistening yellow crystal deposits in the posterior pole and atrophic changes in the RPE. The retinal vessels appear normal. FFA (**G**) shows diffused spiced-salt-shaped hyperfluorescence in the posterior pole. Visual field changes (**H**) indicate paracentral absolute scotoma and decreased VA at 30°. SD-OCT (**I, J**) show the disappearance of the IS/OS band and the EZ. The thickness of the RPE, retina, and choroid indicates thinning. **Patient 7:** Color fundus images (**K**) show sparkling yellowish-white fine spots in the fundus and a submacular hemorrhage in the left eye. FFA (**L**) shows spiced-salt-shaped and map-shaped hypoautofluorescence. The visual field (**M**) shows mild reduced VA at 30°. SD-OCT (**N**, **O**) shows that the structure of the outer retina is complete, with only thinning of the choroid. Note A limited bulge is observed below the foveal in the left eye.

CYP450 family 4. Cytochrome P450s are heme-thiolate proteins involved in the oxidative degradation of various compounds. Sequence conservation is relatively low within the family (there are only three absolutely conserved residues), but their general topography and structural fold are highly conserved. The conserved core is composed of a coil termed the "meander," a four-helix bundle, helices J and K, and two sets of beta-sheets. These constitute the heme-binding loop (with an absolutely conserved cysteine that serves as the 5th ligand for the heme iron), the proton-transfer groove, and the absolutely conserved EXXR motif in helix K [30-35]. In this study, we found eight novel mutations, including two

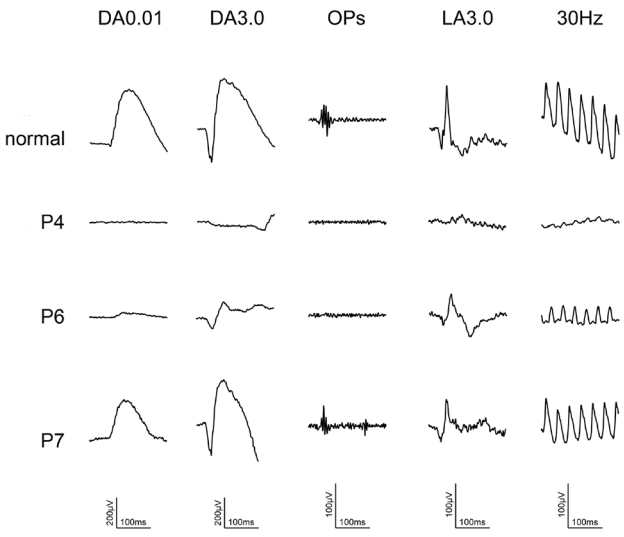

Figure 2. FERGs for three patients with the Hom.c.802–8_810del17insGC mutation. Non-recordable full-field electroretinograms (FERGs) are observed in patient 4, and severely reduced amplitudes on the rod and cone ERGs of patient 6. Normal FERGs were recorded for patient 7.

missense mutations, one nonsense mutation, three deletion mutations, and one splicing mutation. These mutation sites are located within the *CYP4V2* protein domain, and these sites are relatively conserved. The pathogenicity of the nonsense mutations was determined. Deletion mutations c.802–6_810delATACAGGTCATCGCT, c.414_450del, c.492delT, c.796_810del, and c.802–8_807delCATACAGGTCATC lead to misalignment and rearrangement of the encoded amino acids, resulting in protein truncation and functional inactivation. For the mutation c. 1406–1G>A, which has an impact on the protein domain.

Furthermore, a causal link between the mutation in *CYP4V2* and the mechanism(s) causing the BCD phenotype has not been clarified [36-38]. Only a limited number of cases have been reported concerning the relationship between the *CYP4V2* genotype and the BCD clinical phenotype. Lai et al. [10] summarized nine types of mutations in *CYP4V2* in 18 Chinese families, and analyzed the relevant phenotypes. The results showed patients with splice-site mutations (homozygous c.802–8_810del17insGC or compound heterozygous c.802–8_810del17insGC and IVS8–2A>G) had a lower electrooculogram Arden index, and were likely to exhibit a non-recordable scotopic FERG, as well as a non-recordable 30-Hz flicker ERG. However, the influence of the age of onset and the course of the disease factors related to changes in retinal function were not reported. Halford et al. [39] analyzed the genotype and phenotype for 20 patients with BCD from 17 families, and found high variances. Gekka et al. [4] compared clinical features of two cases with a mutation in *CYP4V2*, and found that the patient with the homozygous deletion/insertion mutation (c.802–8_810del17insGC) showed decreased VA, constriction of the visual fields, and severely reduced amplitudes in rod and cone ERGs. Conversely, the other patient with a compound heterozygote mutation, although 6 years older, presented mild clinical manifestations, i.e., satisfactory VA and substantial amplitudes on rod and cone ERGs. Rossi et al. [40] described the clinical and genetic features of 15 Italian patients with BCD, and highlighted the lack of a clear genotype-phenotype correlation underlying the less severity of clinical manifestations that might be linked to relatively mild mutations. In addition, the study described a patient with BCD carrying the mutation in *CYP4V2* and classical retinal lesions, with a normal ERG; however, a multifocal ERG showed that the central P1 wave form was completely extinguished [41]. Yin et al. [42] appraised the genotypes and phenotypes of 17 patients with BCD from 14 families, and found that the phenotype might be variable between different families, although the genotypes were identical.

This is the first study examining the differences in the phenotype among 16 probands with homozygous deletion/insertion mutation, c.802–8_810del17insGC. We found that the same type of mutation resulted in different phenotypes within the group; in particular, the phenotypic analysis in patients within the same age group revealed a high variation between the phenotype and genotype. The severity of the phenotype seems to be related to the course of the disease; the earlier the onset, the longer the duration with a severe phenotype.

Conclusion: We identified seven novel mutations in *CYP4V2* in a population of 85 probands with BCD, thus expanding the spectrum of mutations in *CYP4V2*. The mutations in *CYP4V2* might be widespread in the Chinese population. However, a distinct genotype-phenotype correlation among patients with homozygous deletion/insertion mutations was not observed, suggesting that heterogeneity in genotype and phenotype might underlie BCD. Thus, further studies investigating the possible mechanism(s) underlying these diverse mutations are essential.

APPENDIX 1. FIFTY-SEVEN RP-RELATED GENES BY TARGETED REGION CAPTURE AND NEXT-GENERATION SEQUENCING.

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

APPENDIX 2. CLINICAL FEATURES OF PATIENTS WITH A HOM.C.802–8_810DEL17INSGC MUTATION.

To access the data, click or select the words "Appendix 2." BCVA, best corrected visual acuity; LE, left eye; RE, right eye ; SD-OCT, spectral domain optical coherence tomography; FERG, full-field electroretinogram; NB, night blindness; M, male; F, female; DV, decreased vision; VA, visual acuity; NR, not recordable; ND, not detected.

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