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Identification of novel *CYP4V2* gene mutations in 92 Chinese families with Bietti's crystalline corneoretinal dystrophy

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Purpose: To characterize the spectrum of *CYP4V2* gene mutations in 92 unrelated Chinese probands with Bietti's crystalline dystrophy (BCD) and to describe the molecular and clinical characteristics of four novel *CYP4V2* mutations associated with BCD.
Methods: All study participants underwent a complete ophthalmological examination. Mutational screening of *CYP4V2* coding regions and flanking intron sequences was examined via directional Sanger sequencing, with allele separation confirmed by screening other family members. Subsequent in silico analysis of the mutational consequence on protein function was undertaken, with the impact of the novel mutation on pre-mRNA splicing examined via RT–PCR.
Results: Fifteen disease-causing variants were identified in 92 probands with BCD, including four novel mutations and eleven previously reported mutations. The most prevalent mutation was c.802_810del17insGC, which was detected in 69 unrelated families, with an allele frequency of 52.7% (97/184). Homozygosity was revealed in 35 unrelated families, and compound heterozygosity was observed in 43 subjects. Four patients harbored four novel variants, with these mutations cosegregated within all affected individuals and were not found in unaffected family members and 100 unrelated controls. Transcriptional analysis of a novel splice mutation revealed altered RNA splicing. In silico analysis predicted that the missense variant, p.Tyr343Asp, disrupted the *CYP4V2* surface electrostatic potential distribution and spatial conformation. Among the patients with four novel mutations, genotype did not always correlate with age at onset, disease course, or electroretinogram (ERG) changes, with phenotypic variations even noted within the same genotype.
Conclusions: The c.802_810del17insCG mutation was the most common mutation in the 92 Chinese probands with BCD examined. Four novel mutations were identified, contributing to the spectrum of *CYP4V2* mutations associated with BCD, with no clear link established between disease phenotype and genotype.

Bietti's crystalline corneoretinal dystrophy (BCD, MIM 210370) is an autosomal recessive retinal dystrophy that was first reported by Bietti in 1937 [1]. It is 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 cases had similar crystals in the corneoscleral limbus. Patients with BCD usually present in the second or third decade, and they progress to legal blindness by the fifth or sixth decade of life [2].

The locus of the gene for BCD was mapped to 4q35, with mutations in the *cytochrome P450, family 4, subfamily V, polypeptide 2* (*CYP4V2*, MIM 608614) gene associated with clinical phenotype. The *CYP4V2* gene consists of 11 exons, encodes a 525 amino acid protein, and belongs to the CYP450 family. *CYP4V2* is widely expressed in various tissues, including the human retina, RPE, lymphocytes, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, and has been thought to play a crucial role in fatty acid and corticosteroid metabolism [3,4].

BCD is relatively common in Eastern Asian populations, with *CYP4V2* the only identified gene associated with the disease thus far. At present, 58 mutations have been described, which affect 47 amino acid positions within the protein [4-21]. All of these previously identified *CYP4V2* mutations in patients with BCD were missense coding changes or insertions and/or deletions of one or a few amino acids. Among these mutations, the most common among Chinese patients with BCD include c.802_810del17insGC in exon 7, c.992A>C in exon 8, and c.1091–2A>G in an intronic, accounting for 83.3% of the mutant alleles [14]. To understand the distribution spectrum of these *CYP4V2* mutations in Chinese patients with BCD, 92 unrelated probands were screened, and the molecular and clinical characteristics of novel *CYP4V2* mutations were described.

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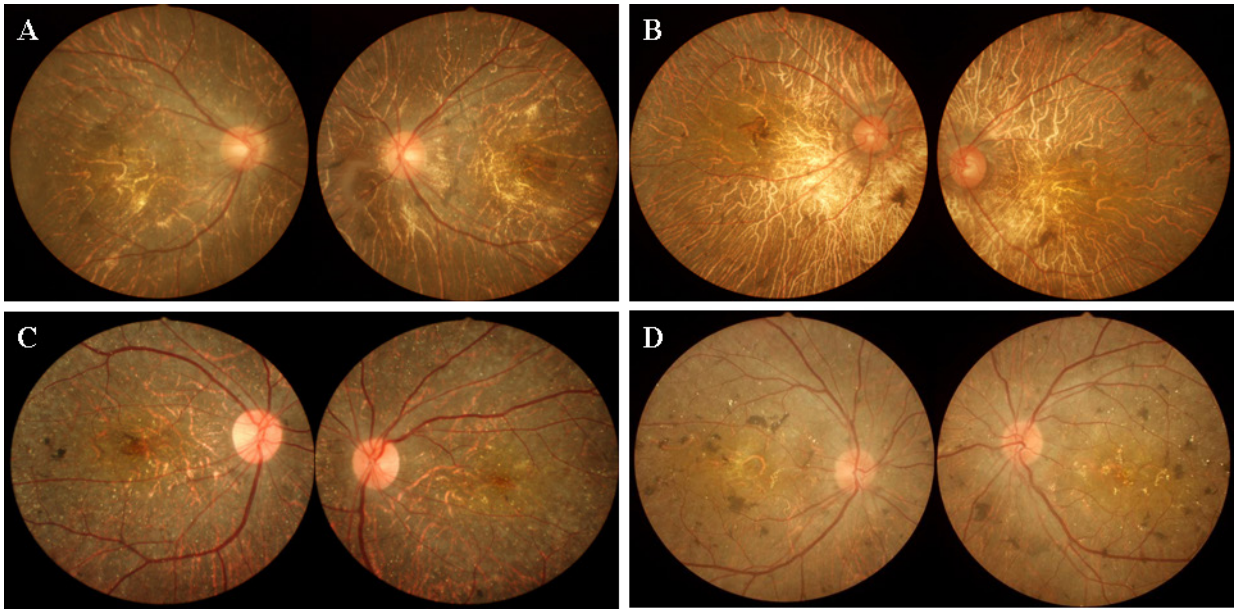

Figure 1. Fundal photographs of four patients with BCD with novel mutations. **A**: Retinography of patient 31. **B**: Retinography of patient 60. **C**: Retinography of patient 33. **D**: Retinography of patient 86.

METHODS

Recruitment of subjects: Ninety-two probands, clinically diagnosed with BCD, from unrelated families at the Southwest Eye Hospital/Southwest Hospital, China, were recruited. Family members of the probands were also clinically examined, in addition to 100 normal controls who were referred for this study. The Ethics Review Board of the Southwest Hospital (Chongqing, China) approved all research protocols, which adhered to the tenets of the Declaration of Helsinki, with informed consent obtained from all participants. All probands underwent ophthalmological examinations including best-corrected visual acuity testing with the Snellen vision chart, slit-lamp biomicroscopy, and fundoscopy. Additionally, fundus photography was performed in 91 patients, automated perimetry (low vision model) in 18 patients, full-field electroretinography (FERG) in 82 patients, which was recorded according to the standards of the International Society for Clinical Electrophysiology of vision (ISCEV, 2008), and multifocal electroretinogram (mfERG) in 77 patients.

Mutation screening/detection: Blood samples were collected from 92 probands and their family members and 100 healthy controls, then preserved in freezers at -80 °C prior to use. Genomic DNA was extracted from whole blood using the

Table 1. Demographics, Clinic data and Genotypes of Patients with novel mutations for the CYP4V2.

Patient	Age/ gender	Age at onset of NB	Age at onset of DV	Refractive error (SE)	VA senllen	Nucleotide change (Mutations)
31	43/F	30	11	RE: —10.5	RE:20/200	c.283G>A, p.G95R+ c.1027T>G, p.Y343D*
				LE:—10.5	LE:20/125	
33	61/F	55	55		RE:20/400	c.802−8_810del17insGC+ c.413+2T>G,del*
					LE:HM	
60	55/M	49	44	RE: —2.75	RE:20/200	c.31C>T, p.Q11X*+ c.31C>T, p.Q11X*
				LE: —2.75	LE:20/125	
86	44/M	41	34		RE:20/200	c.802−8_810del17insGC+ c.791 del T*
					LE:20/50	

NB: night blindness. DV: decrease of visual acuity. RE: right eye. LE: left eye. * Novel mutation.

Tiangen blood kit (Tiangen Biltech, Beijing, China) following the manufacturer's standard sequencing protocols. Genomic DNA was extracted from whole blood using the Tiangen blood kit (Tiangen Biltech) according to the manufacturer's protocols. All *CYP4V2* coding exons, including intron–exon boundaries, were amplified in the 92 probands samples via PCR using primers previously described by Li [12]. The PCR products were subsequently purified with a TIANgen Midi Purification Kit (Tiangen Biltech), sequenced with an ABI BigDye Terminator Cycle Sequencing kit v3.1 (ABI Applied Biosystems, Foster City, CA), and DNA sequences analyzed using the vector NTI 10.3 software package. *CYP4V2* orthologs were also investigated with the NCBI HomoloGene database.

Total RNA was extracted from white blood cells using TRIzol Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. An RNA PCR kit (TaKaRa, Dalian, China) was used to synthesize cDNA from 2 μg of RNA according to the manufacturer's protocols. During the RT–PCR reaction, cDNA was amplified using the primers: RT1-F: 5′-GAA GCC GGA CGG GCG AGA AT-3′, RT1-R: 5′-CTC CTG GAG CGC CAT TTG TTT-3′ to generate *CYP4V2* fragments.

Functional significance prediction: To illustrate the effects of novel *CYP4V2* mutations, the conservation of p.Tyr343, p.Gln11, and p.Phe264 was analyzed among eight species, the molecular structures of novel missense mutation was analyzed in silico, and pre-mRNA splice-site mutations were confirmed with reverse transcription-PCR (RT-PCR).

The crystal structure of *CYP4V2* was modeled based on the crystal structure of CYP46A1, a member of the cytochrome P450 superfamily (PDB ID: 3mdm) and an enzyme that initiates the major pathway of cholesterol elimination from the brain, with an amino acid sequence homology of 25%. A molecular model of *CYP4V2* was constructed with the SWISS-MODEL server, with structural graphics and visualization based on the Swiss-Pdb Viewer 4.04.

RESULTS

Clinical findings: Of the 92 probands, 49 were male and 43 were female, with five consanguineous individuals in the cohort. The affected individuals presented ages of initial

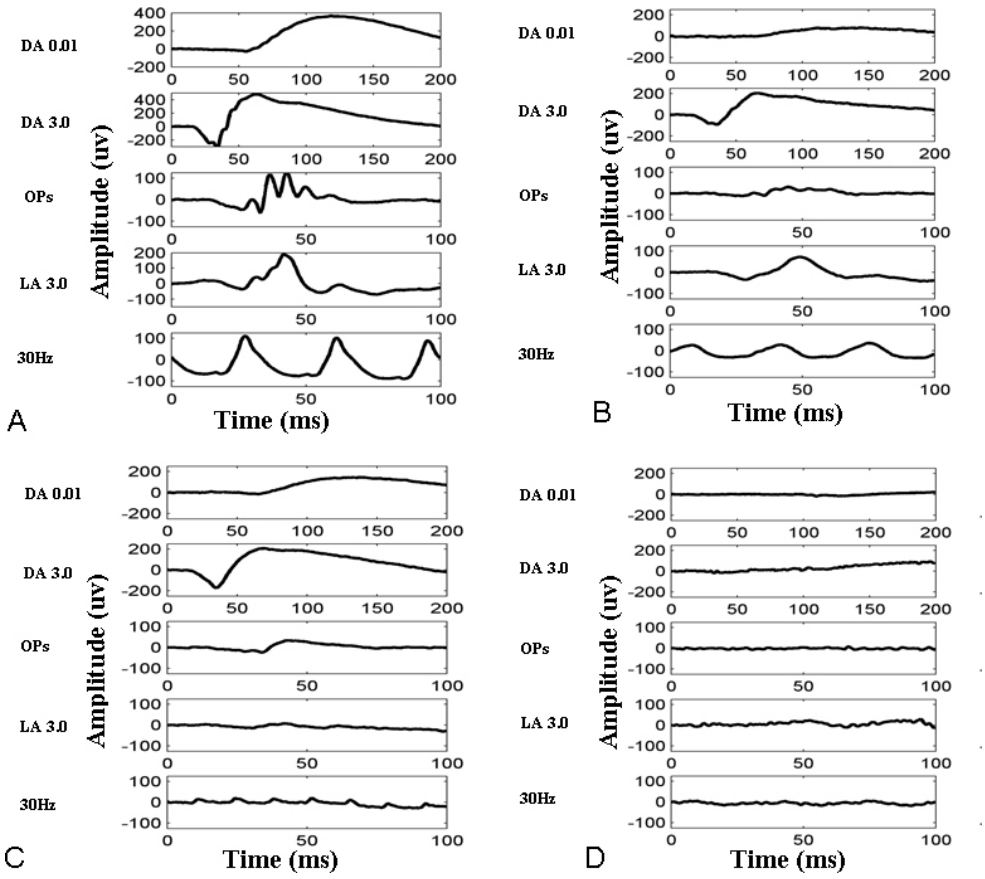

Figure 2. Full-field ERG of normal subjects and BCD patients. **A**: Normal electroretinogram (ERG) from age-matched subject. **B**: Cone dominant ERG. **C**: Rod dominant ERG. **D**: Non-recordable ERG from patient 33, patient 60, and patient 86.

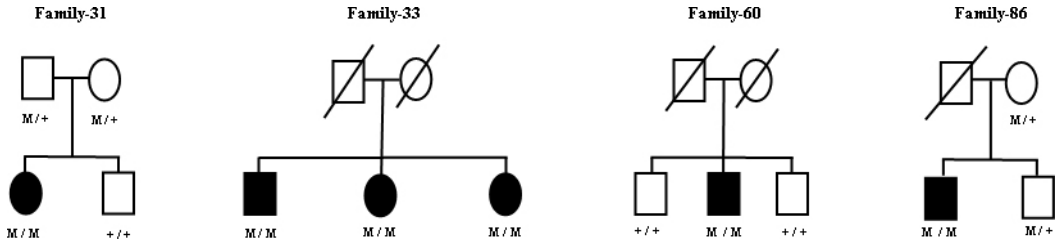

Figure 3. Pedigree and mutation analysis with novel mutation. Men and women are represented by squares and circles, respectively. Filled symbols: affected patients; open symbols: unaffected subjects. Genotypes of family members at the *CYP4V2* gene are indicated. m, mutant allele; +, wt allele.

symptoms of night blindness from 17 to 55, with most individuals in their 30s. Fundus observations revealed typical BCD changes in all 92 probands, including numerous small yellow-white crystalline spots distributed on the macula, posterior poles, and peripheral retina, with the number of crystalline spots varying significantly among patients. Examination of all probands revealed 89 patients (96.7%) with RPE atrophy (age range from 24 to 61), ten patients (10.9%) with severe choroidal sclerosis (age range from 25 to 61), 39 patients (42.4%) with crystals in the limbus cornea (age range from 31 to 62), and 70 patients (76.1%) with vitreous crystals (age range from 20 to 62). Visual acuity varied markedly from light perception (LP) to 20/20, and 61/92 patients (67.8%) showed low vision (best-corrected visual acuity worse than 0.5 logMAR but equal or better than 1.3 logMAR in the better eye, which equals the Snellen chart 20/63). Low visual peripheral (LVP) analysis further showed 14 patients (22 eyes) with irregular visual field defects (mean sensitivity, MS, 10.1 to 26.9) and nine patients (14 eyes) with retained visual islands (MS 2.6 to 10.2). For the ERG recordings, 38 patients (41.3%, 74 eyes) had a non-recordable rod response, only three patients (3.3%, five eyes) showed a rod dominant ERG, and 43 patients (46.7%, 85 eyes) showed a cone dominant ERG. Furthermore, mfERG recordings showed 58 patients (60.9%, 116 eyes) had severe reductions in the response amplitudes of the central and peripheral macula, seven patients (7.6%, 14 eyes) had non-recordable mfERG

Table 2. CYP4V2 mutations identified in 92 families with BCD.

Exon	DNA Change	Amino acid change	Type of nucleotide change	Allele frequency in		Note
			hom/heter	100 Controls	92 probands	
1	c.31C>T	p.Q11X	1/0	0/200	2/184	novel
1	c.64C>G	p.L22V	1/4	ND	6/184	Reported
2	c.215–2A>G	Splicing acceptor	2/0	ND	4/184	Reported
2	c.219T>A	p.F73L	0/3	0/200	3/184	Reported
2	c.283G>A	p.G95R	0/5	ND	5/184	Reported
3	c.413+2T>G	Splicing acceptor	0/1	0/200	1/184	novel
6	c.732G>A	p.W244X	0/1	ND	1/184	Reported
6	c.791del T	delet	0/1	0/200	1/184	novel
7	c.802–8_810del17insGC	Splicing acceptor	28/41	ND	97/184	Reported
7	c.958C>T	p.R320X	0/1	ND	1/184	Reported
8	c.992A>C	p.H331P	0/22	ND	22/184	Reported
8	c.1027T>G	p.Y343D	0/1	0/200	1/184	novel
8	c.1061–1062insA	Ins	0/2	ND	2/184	Reported
9	c.1091–2A>G	Splicing acceptor	3/11	ND	17/184	Reported
9	c.1187C>T	p.P396L	0/2	ND	2/184	Reported
Total			35/95		165/184	

recordings, and only 12 patients (10.9%, 23 eyes) with BCD had mild reductions of mfERG.

The clinical characteristics of the four patients with novel mutations were analyzed, with the age at onset of first symptoms of night blindness and decreased visual acuity ranging from 11 to 55 (Table 1). Visual acuities ranged from 20/50 to hand movement (HM), and all patients had characteristic numerous small retinal crystalline deposits, varying degrees of RPE atrophy, and choroidal sclerosis in the posterior pole. Fundus revealed patient 31, patient 85, and patient 86 had serious choroidal sclerosis (Figure 1). Slit-lamp examination showed two patients had peripheral corneal crystalline deposits. FERG responses were extinct in three patients (patient 31, patient 60, patient 86; Figure 2). Multifocal ERG response was severely reduced in four patients.

Mutation analysis: Following sequence analysis of the *CYP4V2* coding and adjacent intronic regions, homozygous (35 families), compound heterozygous (43 families), and heterozygous mutations (nine families) were detected, with no *CYP4V2* mutation detected in five families. Fifteen mutations were identified in total, with four mutations identified as novel (Table 2).

The c.802_810del17insGC mutation was detected in 69 unrelated families, with an allele frequency of 52.7% (97/184), while the c.992A>G mutation accounted for 11.9% (22/184), and the c.1091–2A>G accounted for 9.8% (17/184). Collectively, these three common mutations comprise 73.37% of the mutations of *CYP4V2*, with c.802_810del17insGC the most prevalent mutation in 92 Chinese families with BCD. The results further demonstrate that mutation c.802_810del17insGC is one of the most common mutations in Chinese populations.

Four non-consanguineous patients had four novel mutations, which have not been previously reported (Figure 3). The underlying novel variations revealed three kinds of compound heterozygosity and one homozygous mutation in the four patients. The novel mutations were cosegregated within all affected individuals and were not found in the 100 Chinese control individuals. A missense mutation, c. 1027T>G, was identified in patient 31 (Figure 4A,B), which

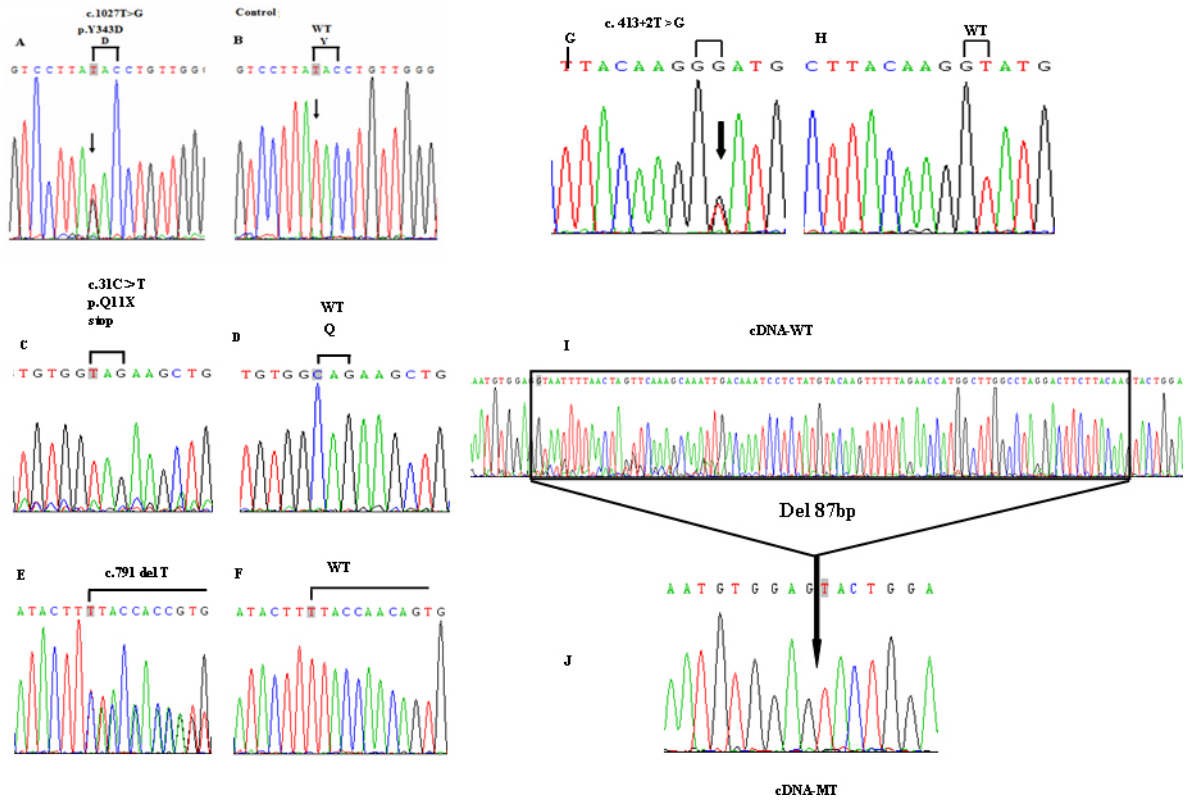

Figure 4. Genomic DNA and cDNA of the *CYP4V2* gene in four novel mutations. **A**: The heterozygous mutant single base-pair change (c.1027T>G,p.Y343D). **B**: Control (wild type, WT). **C**: The homozygous nonsense mutant (p.Q11). **D**: Control (WT). **E**: The heterozygous change (c.791del T). **F**: Control (WT). **G**: DNA-MT of the heterozygous splice change (c.413+2T>G). **H**: Control (WT). **I**: cDNA sequence, from the sense primer, for the control (WT). **J**: The cDNA of the heterozygous splice change (c.413+2T>G). The deletion is indicated with an arrow and black frame.

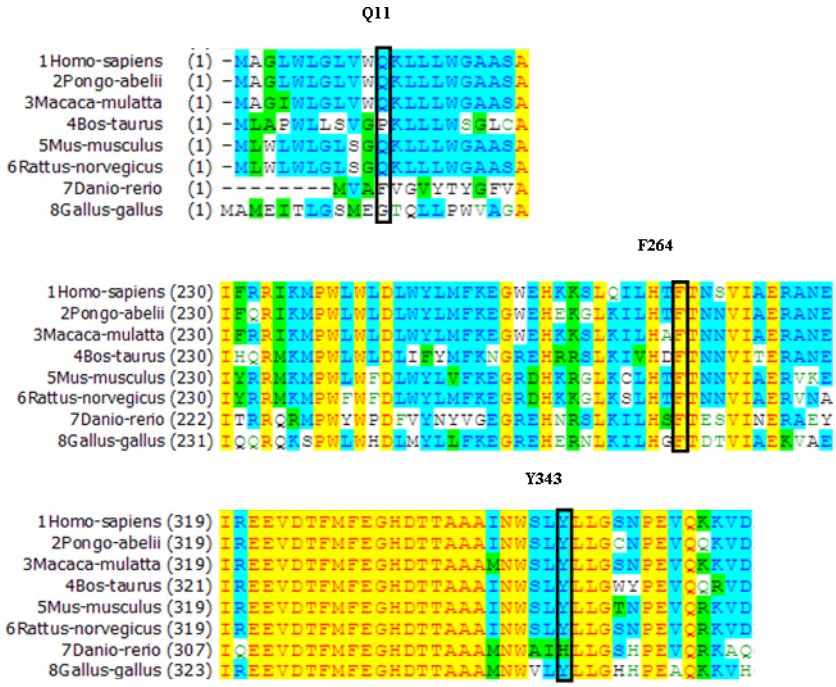

Figure 5. The alignment of novel mutations of the *CYP4V2* gene with the corresponding segments in eight species. Amino acids marked in the column are highly conserved among all shown species in F264, while Q11 is conserved among five shown species and Y343 is conserved among seven shown species. Background color illustration: transparent=non-similar; light blue=conserved; yellow=identical.

resulted in a tyrosine to aspartic acid change (p.Tyr343Asp). In patient 60, a nonsense mutation (c.31C>T) that converts a glutamine (CAG) into a premature termination codon (TAG) at amino acid position 11 (p.Gln11X) in exon 1 was identified (Figure 4C,D), resulting in the conversion of a 525-amino acid sequence to an 11-amino acid (MAGLWLGLVWQ) sequence. A heterozygous deletion, c.791delT, which causes the deletion of an amino acid at position 264, resulting in an in-frame shift, was identified in patient 86 (Figure 4E,F). In patient 33, one novel heterozygous splice-site mutation, c.413+2T >G, was detected in the 3′- acceptor in intron 3, resulting in a GTA to GTA (Figure 4G,H). Furthermore, RT–PCR showed that the cDNA nucleotide sequence derived from the mutant transcript resulted in a truncation of exon 3, resulting in a 87-bp/29-amino acid deletion (Figure 4I,J).

Conservation of novel mutations in CYP4V2: *CYP4V2* encodes a 525-amino acid protein that belongs to a member of the *CYP450* family and is widely expressed in human tissues including the sensory retina and the RPE. A *CYP4V2* ortholog investigation revealed that p.F264 was highly conserved among eight species although p.Q11 and p.Y343 were not completely conserved across all eight species (Figure 5).

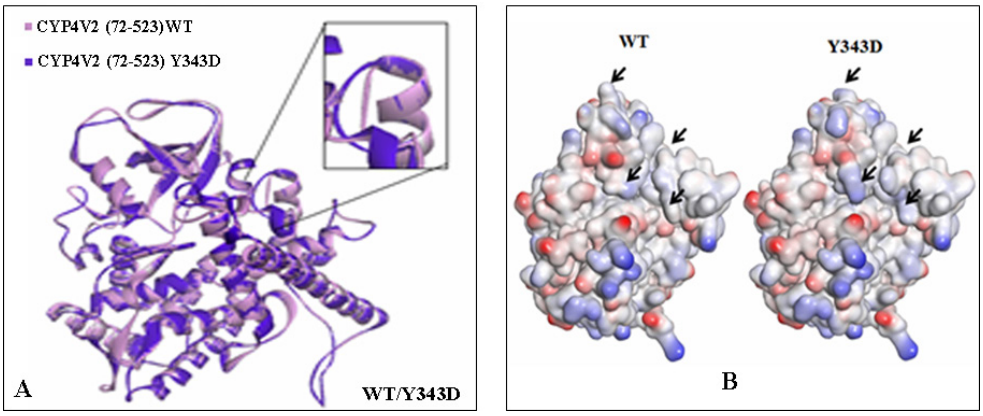

Figure 6. Superimposed wild-type and mutant *CYP4V2* and electrostatic potential differences between WT and Y343D. **A**: Wild-type *CYP4V2* (72–523; pink) and *CYP4V2* (72–523) Y343D (blue) is shown on the right. The typically disturbed secondary structures are magnified for detail. **B**: Blue denotes a positive charge, and red denotes a negative charge. The arrows indicate the charge distribution change region on the molecular surface in the mutants and the wild type (WT).

Mutant structure modeling of one missense mutation: Mutant molecular model showed that p.Tyr343Asp affected the alpha spiral structure on the surface region of the protein (Figure 6A), and disrupting the surface electrostatic potential distribution and the spatial conformation of *CYP4V2* (Figure 6B).

DISCUSSION

In this study, a total of 15 *CYP4V2* mutations were identified from the 92 probands with BCD and 92 unrelated families of Chinese ethnicity; four were novel pathogenic variants. Thus far, this is one of the largest BCD-associated *CYP4V2* mutational screenings in a Chinese population. Among the 15 mutations, the nucleotide deletion/insertion c.802–810del17insCG was the most frequently occurring pathogenic mutation, which could be a common founder effect contributing to the high prevalence in the Chinese population. The c.992A>G and c.1091–2A>G mutations were also common in this study, with these findings in agreement with previous studies [4,8,12,14].

In our cohort, four novel mutations were identified in the *CYP4V2* gene, with targeted mutational analysis of family members confirming that these four novel mutations, p.Tyr343Asp, c.413+2T>G, c.791delT, and p.Gln11X, segregated with the BCD phenotypes and were absent from a panel of 100 normal unrelated controls. These four novel mutations may affect the structure and function of *CYP4V2*, such as the c.413+2T>G mutation that was located in a splice site and led to a protein truncation. This deletion of 87 bp, resulting in the deletion of 29 amino acids, must affect the structural stability and function of *CYP4V2*. Second, a novel nonsense mutation (p.Gln11X) in exon 1 causes premature termination and eliminates a large portion of the *CYP4V2* structure. Third, due to a frameshift deletion mutation, c.791delT in exon 6, the amino acid sequence was altered to create a false sequence, resulting in an abnormal protein structure and a nonfunctioning *CYP4V2*. For the novel heterozygous missense mutation, in silico analysis revealed that p.Tyr343, amino acid substitutions not only changed the local random coil structure on the surface of *CYP4V2* but also disrupted the surface electrostatic potential distribution and space conformation. Although mutation, p.Tyr343, did not completely at a highly conserved site in cytochrome P450, it is a low frequency mutation in *CYP4V2*, and database for nonsynonymous SNPs' functional predictions (dbNSFP) prediction showed that p.Tyr343 is a damaging and disease-causing mutation. However, further investigation is needed to elucidate the pathogenic mechanisms of the novel missense mutations.

Earlier reports have suggested that different *CYP4V2* mutations could cause divergent BCD phenotypes [9,10,12,17,18]. In this study, we described the genotype-phenotype correlations for four patients with novel mutations. The phenotypes consisted of varying degrees of crystalline deposits, choroidal sclerosis, RPE atrophy, age of onset, disease course, and ERG changes, with severity not always associated with genotype. Patient 60, who had a deleterious homozygous novel nonsense mutation (p.Q11X), did not exhibit earlier disorder symptom and disease severity relative to the patients with heterozygous missense mutations or splice mutations.

Patient 33, who harbored a deletion/insertion mutation (c.802–8_810del17insGC) and a novel splice mutation (c.413+2T>G), exhibited a late age of onset, rapid disease progression, and severe vision loss compared with other patients with splice mutations

in our cohort. It was also discovered that ERG response in these patients did not seem to be associated with the type of mutation. Among the four patients with novel mutations, FERG was extinct in four patients, and the mfERG response was severely reduced in four patients with different mutations, which is different from previous reports [12]. Moreover, age of onset did not always correlate with genotype severity. For example, patient 31, who had compound heterozygous missense mutations (G95R and Y343D), exhibited early onset, had a relatively slow disease progression, and presented with similar severe morphofunctional changes as those seen in patient 60 (Q11X), who exhibited late age of onset and fast disease progression. Furthermore, the age of onset varied among patients with the same mutations, further supporting the high variability between the BCD genotype and phenotype. This suggests that the clinical phenotype severity in patients with BCD cannot be determined by genotype; thus, other factors such as gene modifications, gene regulation, and environmental factors may influence the interchange between pathogenicity and *CYP4V2* mutations.

Interestingly, this study discovered seven patients (8.9%) with BCD exhibited high myopia, which has not been reported in other Chinese families with BCD. There was no evidence that this myopia directly accelerated BCD progress in our primary study.

We observed that patients with high myopia appeared to have only poorer best-corrected visual acuity and didn't show more affected retinal functioning based on ERG response. Due to the lack of continuous observation of retinal function changes of continuous and binocular disparity, it is difficult to confirm that the ERG responses in these patients with BCD associated with high pathological axial myopia

showed additional decreases. In fact, the amplitude of the a wave and the b wave on ERG decreased in the axial high myopia eyes (ocular axial length is more than 26 mm, equivalent diopter (D) greater than 6.25D). Retinal degeneration diseases combined with high myopia are rare, with some studies suggesting a compounding effect, but currently, there is not enough evidence to prove a correlation with the retinitis pigmentosa (RP) clinical phenotype [22-25]. Further observation is needed to determine if the combined ocular complications in BCD aggravate retinal function.

In conclusion, the c.802_810del17insCG mutation was found to be the most common mutation in 92 Chinese probands with BCD. Four novel mutations were identified, contributing to the spectrum of *CYP4V2* mutations associated with BCD, with no clear link established between disease phenotype and genotype.

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