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Novel mutations in the *CYP4V2* gene associated with Bietti crystalline corneoretinal dystrophy

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Purpose: Bietti crystalline corneoretinal dystrophy (BCD) is an autosomal recessive disorder of retinal degeneration characterized by small glittering crystals in the corneal limbus, posterior pole of the eye, and circulating lymphocytes. Recently mutations in a new gene *CYP4V2*, encoding a protein belonging to a novel member of the cytochrome P450 family, have been identified as the cause of BCD. To further characterize the role of *CYP4V2* in BCD, mutation screening has been undertaken in a cohort of affected patients with BCD from China.

Methods: Eight unrelated families, including 14 patients and 18 unaffected relatives, and 10 sporadic patients were examined clinically. Fifty normal Chinese individuals served as control subjects. Genomic DNA was extracted from venous blood of all participants. The coding region (including the intron-exon boundary) of *CYP4V2* was amplified by polymerase chain reaction (PCR). The PCR products were analyzed using direct sequencing and single strand conformation polymorphism (SSCP).

Results: Fundus examination revealed clinical features of BCD with many small, yellowish-sparkling crystals at the posterior pole of the fundus. Sequencing of *CYP4V2* identified nine (5 missense, 1 nonsense, 2 deletion, and 1 point A->G transversion in the splice accepter site) mutations in 8 families and 9 independent patients. Five of these mutations are novel.

Conclusions: Our finding expands the spectrum of *CYP4V2* mutations causing BCD, and further confirms the role of *CYP4V2* in the pathogenesis of BCD.

Bietti crystalline corneoretinal dystrophy (BCD) is an autosomal recessive disorder of retinal degeneration characterized by numerous tiny sparkling yellow-white spots at the posterior pole of the fundus. The small glittering crystals can also occur in the corneal limbus and circulating lymphocytes [1-3]. Clinically, affected patients experienced decreased vision, night blindness, and constriction of the visual fields, which usually occurs around the third and forth decade of life [2,3]. BCD is a rare retinal disorder worldwide, however it appears to be relatively common in China and Japan, where the gene frequency has been estimated to be 0.005 [4,5].

Histologically, BCD shows evidence of advanced panchorioretinal atrophy characterized by generalized loss of and sclerosis of the choriocapillaris with crystals and complex lipid inclusions within the choroidal fibroblasts. The abnormal inclusions are similar to the ones found in circulating lymphocytes, keratocyte, and conjunctival and skin fibroblasts [2,3]. According to these data, BCD may be from the systemic abnormalities of lipid metabolism. Cultured lymphocytes from BCD patient were detected to lack two fatty acid-binding proteins of 32 and 45 kDa, which presented in age-matched controls [6]. Further study showed cultured lymphocytes and fibroblast from BCD patients had abnormally high levels of triglycerides and cholesterol storage, consistent with the finding of low conversion of FA precursors into n-3 PUFA [7].

BCD had previously been linked to chromosome 4 (4q35) [8]. Recently, mutations in a new gene *CYP4V2* have been identified as the cause of BCD [9]. The coding region of the *CYP4V2* gene spans approximately 19 kb, consisting of eleven exons that code for a 525 amino acid polypeptide which is homologous to other members of CYP450 family 4. The gene is expressed widely in human heart, brain, placenta, lung, liver, retina, and RPE. To date, 16 mutations have been described in patients with BCD [9-11].

In this study, we performed mutation screening of *CYP4V2* gene in 8 Chinese families and 10 sporadic individuals with clinical diagnosis of BCD and identified nine mutations, five of which were novel.

METHODS

Clinical data and sample collection: This study was granted approval from the Beijing Tongren Hospital Joint Committee on Clinical Investigation and conformed to the tenets of the Declaration of Helsinki. Eight unrelated Chinese families and ten separate individuals with BCD were referred to Beijing TongRen Hospital. After informed consent was obtained, all participants underwent full ophthalmologic examination including best corrected visual acuity, slit-lamp, and fundus examination with dilated pupils. Ophthamoscopic findings were recorded by color fundus photography. The proband of each family and most sporadic patients underwent fundus fluorescein angiography (FFA), electroretinography (ERGs), and vi-

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sual field examination. Clinical diagnosis was based on the presence of many tiny sparkling yellowish crystals in the posterior pole, atrophy of retinal pigment epithelium, and sclerosis of the choriocapillaris.

Mutation detection: Blood samples were obtained by venipuncture, and genomic DNA was extracted according to standard protocols. The coding region of *CYP4V2* was amplified by polymerase chain reaction (PCR). Ten pairs of primers were used for the coding region of *CYP4V2* as described by Li et al. [9] (Table 1). For direct sequencing, PCR products were purified (Shenneng Bocai PCR purification kit; Shenneng, Shanghai, China). Briefly, the PCR products were mixed with the binding buffer, and then the mixtures were processed through the spin column. After being washed two times, the pure DNA was eluted with a small volume of water. The purified PCR products were sequenced using an auto-

TABLE 1. PCR PRIMERS USED IN THIS STUDY

	Sequence (5'-3')	
Primer	Forward	Reverse
Exon 1	AACCTCGCAGCACCCTCAGAA	ACTTTGGGATGGGGCACTAGCAGT
Exon 2	ACCTGGCTTCCTCTAACAGTAACA	TTTTTGTGCTGAAATGGCTGAA
Exon 3	AGATTCGCCTCCTCCCACCTCAC	ACCTGGACTCTTGGCCTCTTGACG
Exon 4	ACCTGGACTCTTGGCCTCTTGACG	CGCGCTGAAGAGCCCGTCAC
Exon 5	AGGAAGAACAGGAACAGGGAGTAG	CAACGCAGAAATTGTTAGCAATAA
Exon 6	GCTTCATGGGATGCGTAATAGC	GAAATGAACGGTGGGGATGGT
Exon 7	CCTATGTTGTCGAAATGTTGAAAT	CCTATGTTGTCGAAATGTTGAAAT
Exon 8	TTGCAGTCACAGTGCAGTCATCA	CCAGCATCCGGCCTAGTACAGTC
Exon 9, 10	CAGCATCCGGCCTAGTACAGTC	TGGGCAATGTCACATCACATCTCA
Exon 11	CTCTTCATCTTTAACAGGTGTTCC	CAAAACTCAAAACTTTTTCTTTGT
Sscp Exon 1	CTCGTGTGGCAGAAGCTG	ACTTTGGGATGGGGCACTAGCAGT
Sscp Exon 3	CAGGAAGGTTGTTTGATGTCTG	CCTGCTACAGAAAAAGCTACCC
Sscp Exon 9	ACCGTCCCGCTACAGTAGAA	AGACCGGAGCAAGGTAGACA

PCR primer sequences (both forward and reverse) for 11 different exons and 3 Sscp of the *CYP4V2* gene are shown.

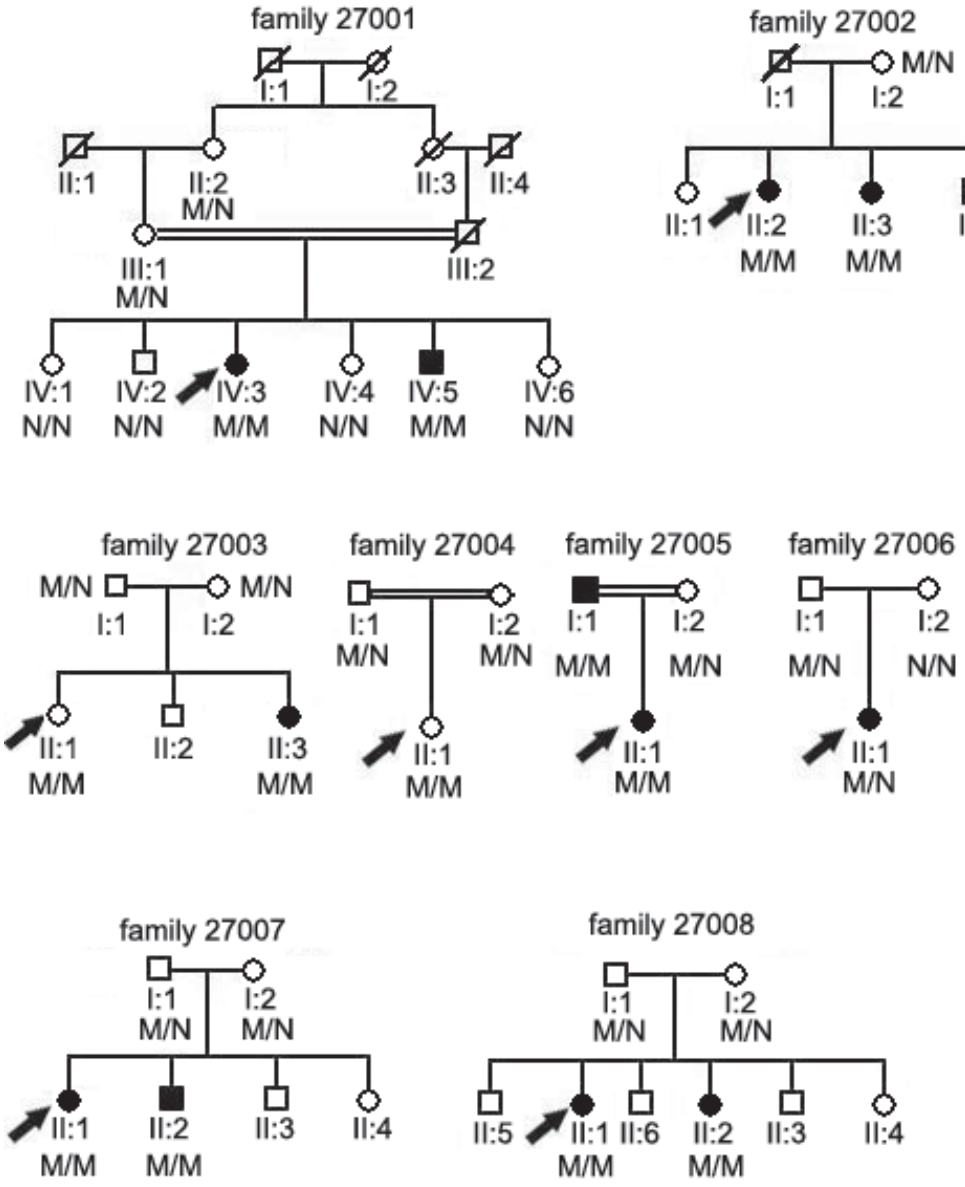

Figure 1. Family structure of the eight Chinese families with Bietti crystalline corneoretinal dystrophy. Pedigrees of eight Chinese families with Bietti crystalline corneoretinal dystrophy associated with the mutations in *CYP4V2*. Males and females are represented by squares and circles, respectively. The symbols for affected family members are filled; the symbols for deceased family members have a slash. Where known, the symbol has been annotated to indicate whether alleles are mutant (M) or normal (N).

matic fluorescence DNA sequencer (ABI, Prism 373a; Perkin Elmer, Foster City, CA), according to the manufacturers' instructions. Nucleotide sequences were compared with the published cDNA sequence of *CYP4V2* (GenBank accession number NM_207352).

Single strand conformation polymorphism (SSCP): SSCP was used to exclude point mutations from normal controls. Amplified DNA was mixed with an equal volume formamide buffer (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). Denatured samples were electrophoreses on a 14% nondenaturing polyacrlamide gel (acrylamide:bisacrylamide=49:1) for 12-16 h at 300 v and 4 °C. After electrophoresis, gels were silver-stained and analyzed [12]. As the PCR fragments used in SSCP analysis were between 150-300 bp, three pairs of specific primers were designed for detecting mutations in exon 1, exon 3, and exon 9 (Table 1).

RESULTS

We have identified 8 unrelated Chinese families and 10 isolated patients with a clear diagnosis of BCD. The inheritance pattern in these 8 families appears to be autosomal recessive and there is a feature of consanguinity in three families (Figure 1). After careful ophthalmologic examination 14 members from 8 unrelated families and 10 sporadic individuals presented with Bietti crystalline dystrophy. Fundus examination with dilated pupil of the affected individuals showed numerous tiny glittering crystals at the posterior pole of the fundus with different extent of atrophy of RPE and sclerosis of the choriocapillaris (Figure 2). No crystals were observed in the cornea and corneal limbus of all affected individuals by slit lamp examination.

Sequencing of *CYP4V2* within the coding region including intron-exon boundaries revealed nine sequence variants in eight unrelated families and 9 isolated individuals with BCD (Table 2). Five of these variants were detected for the first time in our study. The mutations found in the familial cases were confirmed in all affected family members to co-segregate with BCD.

Two homozygous mutations were detected in four families and six isolated patients. A 17 bp homozygous deletion mutation of IVS6-8del TCATACAGGTCATCGCG/insGC, including the exon 7 splice-acceptor site, were detected in three families (27001, 27005, and 27008) and five individuals (28038, 28041, 28042, 28044, and 28051). This mutation results in an in-frame deletion of exon 7, which encodes 62 amino acids. Another homozygous variant, the nucleotide change C367G at codon 22 (CTT->GTT) resulting in a leucine to valine (L22V) change, was identified in family 27004 and patient 28043 (Figure 3A).

Table 2. Mutations identified within *CYP4V2* in BCD-affected families and patients

DNA Change	Exon	Amino acid change	Type of nucleotide change	Family and patient numbers
367C->G	1	L22V	Homozygous	27004, 28043
557C->T	2	R85C	Heterozygous	27003
587G->A	2	G95R	Heterozygous	27003
639T->G	3	L112X	Heterozygous	27001, 28029
*IVS6-8delTCATACA-GGTCATCGCG/insGC (3'[acceptor]splice site)	7	Exon7del	Homozygous	27005, 27008, 28038, 28041, 28042, 28044, 28051
			Heterozygous	27002, 27006, 27007, 28045, 28049
*1296A->C	8	H331P	Heterozygous	28049
*IVS8-2A->G (3'[acceptor]splice site)	9	Exon9del	Heterozygous	27002
1503G->A	9	R400H	Heterozygous	27007
IVS9-6delTGACAGCAGGTTACAG (3'[acceptor]splice site)	10	Exon10del	Heterozygous	28029

Different mutations identified within *CYP4V2* in BCD-affected families and sporadic patients. The family and patient numbers can be distinguished by their first digits, "27" for families and "28" for patients. The asterisks indicate that the mutation has been identified previously by Li et al. [9] and Wada et al. [10].

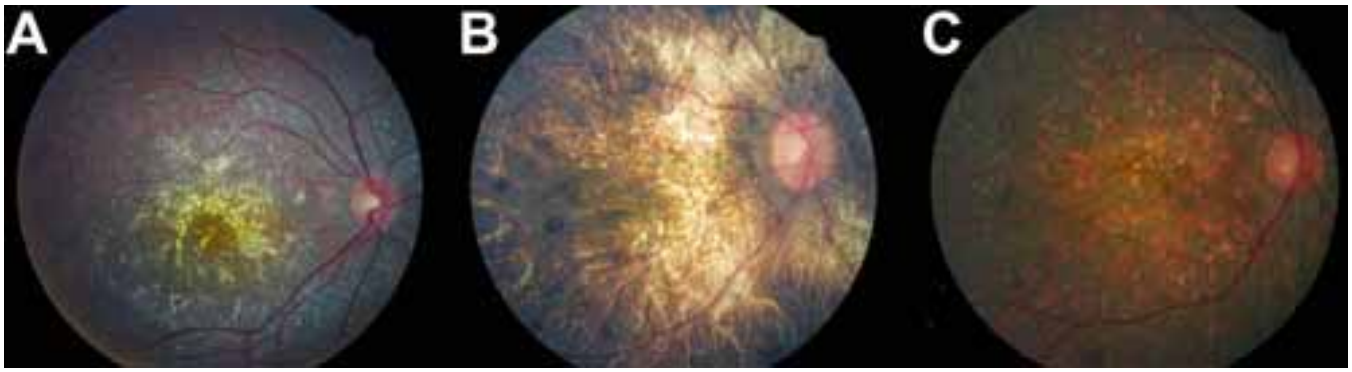

Figure 2. Fundus appearance of the patients with Bietti crystalline corneoretinal dystrophy. **A**: The fundus of the proband of family 27001 has many small yellowish-white sparkling spots distributed in the posterior pole with most of them located at the macular. **B**: The fundus of the proband of family 27002 has severe choroidal sclerosis with a few crystalline deposits. Pigmentation is observed in the peripheral area of the fundus. **C**: Patient 28029 has numerous yellow sparkling crystalline deposits evenly distributed in the posterior pole with pigmentation in the peripheral area.

Five compound heterozygous mutations were detected in three families (27002, 27003, and 27007) and two patients (28029 and 28049). In families 27002, 27007, and patient 28049, one allele harbored a deletion mutation IVS6-8del TCATACAGGTCATCGCG/insGC, the other allele carried a IVS8-2 A->G change resulting in skipping of exon 9, a novel G1503A change at codon 400 (CGT->CAT) resulting in an arginine to histidine (R400H; Figure 3D), and a A1296C change at codon 331 (CAC->CCC) resulting in a histidine to proline (H331P), respectively. However, only one heterzygous deletion mutation, IVS6-8del, was found in family 27006 and patient 28045. In patient 28029, a novel compound heterozygous mutation was detected, of which one allele harbored a 16 bp deletion mutation of IVS9-6del TGACAGcaGGTTACAG including the exon 10 splice-acceptor site, resulting in deletion of exon 10, which encodes 60 amino acids (Figure 3E). The other allele carried a novel T639G change at codon 112 (TTA->TGA), resulting in a leucine to stop (L112X) nonsense mutation (Figure 3C). Another novel compound heterozygous mutation was identified in family 27003. The first heterozygous change, a C557T transition at the first nucleotide position of codon 85, results in an arginine to cysteine (R85C). The other change, a G587A transition also at the first nucleotide position of codon 95, results in a glycine to arginine (G95R; Figure 3B). Fifty normal controls were screened for the novel sequence variants detected in this study. Only a heterozygous C->G change in exon 1 (367C->G, L22V) was found in 22 normal controls. None of the other novel sequence variants was detected in 50 Chinese normal controls.

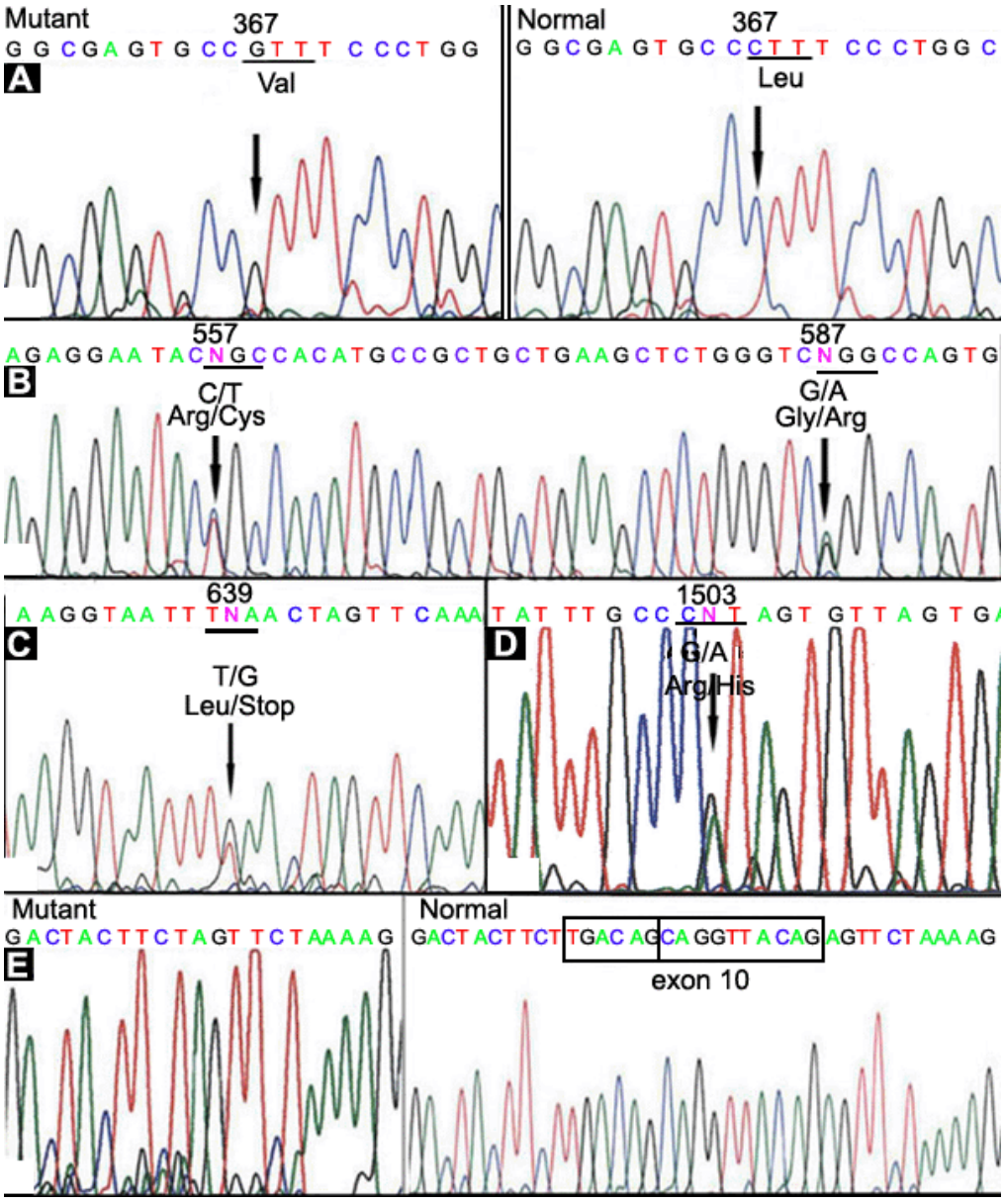

Figure 3. Direct sequencing analysis of the coding region of *CYP4V2*. **A**: The left side shows the homozygous mutant single-base pair change (CTT->GTT, L22V) and the right side shows the corresponding normal sequence. **B**: Heterozygous changes (CGC->TGC R85C and GGG->AGG G95R). **C**: Heterozygous sequence variant (TTA->TGA L112X). **D**: Heterozygous single-base pair change (CGT->CAT, R400H). **E**: The left side shows a 16 bp deletion mutation (IVS9-6del TGACAGCAGGTTACAG including exon 10 splice-acceptor site) and the right side shows the corresponding normal sequence. The deleted nucleotides are in the frame. All sequences above are in the sense direction.

DISCUSSION

In this study, we collected 8 Chinese families and 10 unrelated individuals with clinically diagnosed BCD and screened the *CYP4V2* gene, which is lately reported to be the disease-causing gene of BCD. We have identified nine mutations in seven families and 8 isolated patients, five of which are novel. These include seven point mutations and two deletion mutations.

As showed in Table 2, four families and six individuals in this study carried homozygous mutations, three families and two separate individuals harbored compound heterozygous mutations of the *CYP4V2* gene. This is consistent with a pattern of autosomal recessive inheritance of BCD. However we failed to detect any mutation of *CYP4V2* in one isolated patient (28046) and identified only a single heterozygous mutation in one family (27006) and one individual (28045). It is possible that some mutations are located in the promoter or in a noncoding regions of the gene.

CYP4V2 is a new gene identified recently [9]. It encodes a protein belonging to the P450 heme-thiolate protein super family. Based on homology modeling, the CYP4V2 structure is predicted to have a transmembrane segment residing near the amino terminus, followed by a globular structural domain typical of the CYP450 family. The globular domain of CYP4V2 consists of 18 helices and β-structural segments [9]. Both the novel deletion, IVS9 6del TGA CAG CAG GTT ACA G, and the nonsense mutation, L112X, which eliminate a large part of the CYP4V2 protein, may cause significant changes in the primary protein structure.

Despite the mutations causing loss of a large part of the protein, most of the amino acid substitutions (point mutations) occur at highly conserved sites and result in a significant change in polarity or charge. Two heterozygous missense mutations, G95R and R400H, occur at highly conserved sites across cytochrome P450. G95R substitution results in a significant change in polarity and charge (from no polar to positive polar). Another mutation, R400H, replaces a positively charged arginine with a hydrophobic aromatic histidine. R85C involves a substitution of a non polar for a positive polar residue at a position that is not highly conserved. The substitution of cysteine for arginine, resulting in insertion of a potential disulfide bonding site, is likely to have profound consequences on protein structure [9].

One sequence variant, L22V, was in homozygous in one family (27004) and one patient (28043), and heterozygous in 22 normal controls. As this substitution has been previously reported in an SNP database and was found in patients with other pathogenic recessive mutations in *CYP4V2* [11], it is more likely to be a nonpathogenic sequence variant.

Among the nine mutations of the *CYP4V2* gene identified in this study, the IVS6 8delTCATACAGGTCATCGCG/insGC mutation was detected as homozygous in three families and 5 isolated patients. This allele was also heterozygous in three families and two isolated patients. Recently, Wada and associates [10] identified this mutation in five Japanese families with BCD. More recently Lin et al. [11] detected this mutation in seven unrelated Japanese and Chinese patients. Actually, Li and associates [9] first detected this 17 bp deletion in seven Japanese families and three Chinese families with BCD, which was described as a 15 bp deletion. All these suggest that the IVS6 to 8del TCATACAGGTCATCGCG/insGC mutation is a common mutation in Chinese and Japanese patients with BCD.

No crystalline deposits were observed in the cornea and corneal limbus in this group of patients. It has been reported that cornea crystalline deposits are not present or mentioned in half of the reported cases of BCD [3]. One possibility for this is that Bietti crystalline corneoretinal dystrophy appears to be a progressive condition and the corneal crystals may diminish with time just like the phenomenon occurring in the retina [3]. The other possibility is that the corneal crystalline deposits in some patients are very subtle and they can be detected clearly only by specular microscopy [11].

CYP4V2 is a novel gene and it might play a role in fatty acid and corticosteroid metabolism, however the precise way in which mutations of CYP4V2 cause BCD represents the next challenge in understanding the basis of CYP4V2-mediated retina degeneration. In this study, we performed *CYP4V2* mutation screening and identified nine mutations (including five novel). Our finding expands the spectrum of *CYP4V2* mutations in BCD and further confirms the role of *CYP4V2* in the pathogenesis of BCD.

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