

Characterization of a novel mutation in the *CRYBB2* gene associated with autosomal dominant congenital posterior subcapsular cataract in a Chinese family

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Purpose: To identify the underlying genetic defect in four generations of a Chinese family affected with bilateral congenital posterior subcapsular cataracts.

Methods: Clinical data from patients in the family were recorded by slit-lamp photography. Genomic DNA samples were extracted from peripheral blood of the pedigree members. Mutation screening was performed in the candidate gene by bidirectional sequencing of the amplified products. The mutation was verified by restriction fragment length polymorphism (RFLP) analysis.

Results: The congenital cataract phenotype of the pedigree was identified as posterior subcapsular by slit-lamp photography. Sequencing of the candidate genes detected a heterozygous c.5C→T change in the coding region of the β B2-crystallin gene (*CRYBB2*), resulting in the substitution of a highly conserved alanine to valine (p. A2V). All nine family members affected with cataracts were positive for this change, but it was not observed in any of the unaffected members of the family. The transition resulted in the loss of a HaeIII restriction site in the affected members of the pedigree, which was present in the unaffected family members and in all of the 100 unrelated individuals tested.

Conclusions: This study has identified a novel *CRYBB2* gene mutation, resulting in the amino substitution p. A2V in a Chinese family with posterior subcapsular congenital cataracts. This mutation is probably the causative lesion for the observed phenotype in this family.

Congenital cataracts, the loss of eye lens transparency, are a leading cause of visual impairment or blindness in childhood. Depending on regional socioeconomic development, its prevalence varies from 1–6 cases per 10,000 live births in industrialized countries [1,2] to 5–15 per 10,000 in the poorest areas of the world [3,4]. Globally, congenital cataracts account for approximately one-tenth of childhood blindness due to different causes including infections during embryogenesis, metabolic disorders (galactosemia), and gene defects [5]. As inherited cataracts correspond to 8–25% of congenital cataracts [6], genetic mutation is likely the most common cause. Although autosomal recessive and X-linked forms of inheritance also exist, autosomal dominance is the major form of inheritance of congenital cataracts [7]. Autosomal dominant congenital cataracts (ADCC) have been reported to be caused by mutations in 24 different genes to date: approximately half of the mutations are in the crystallin genes and a quarter in connexins genes, with the remainder divided among the genes for heat shock transcription factor-4 (*HSF4*), aquaporin-0 (*AQP0*, *MIP*), paired-like homeodomain 3 (*PITX3*), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (*MAF*), chromatin modifying protein

(*CHMP4B*), lens intrinsic membrane protein 2 (*LIM2*), beaded filament structural protein-2 (*BFSP2*) [5,8], the forkhead box protein E3 (*FOXE3*) [9], Drosophila eyes absent gene 1 (*EYAI*) [10], intermediate filament protein vimentin (*VIM*) [11], and carboxylic acid transporter family SLC16A12 [12].

According to the location of the lens opacities, and a detailed description of the shape and appearance, a comprehensive approach is to classify the complex spectrum of morphological variations of congenital cataracts. The cataract phenotype can be divided into the following categories: total, nuclear, cortical, anterior subcapsular, posterior subcapsular, lamellar, cerulean, pulverulent, sutural, coralliform, wedge-shaped, and polymorphic cataracts [5, 13]. Mutations in different genes may be associated with similar phenotypes due to genetic heterogeneity [14]. To date, congenital posterior subcapsular cataracts have been related to these genes: *CRYAB* [15,16], *CRYBA1* [17], *GJA8*, receptor tyrosine kinase gene (*EPHA2*) [18], *PITX3* [19], and *CHMP4B* [20].

In this paper, a four-generation family affected with congenital posterior subcapsular cataracts was investigated in an attempt to identify the genetic defect associated with their cataract phenotype.

METHODS

Clinical evaluation and DNA specimens: The four generation of the family suffering with ADCC were recruited from the

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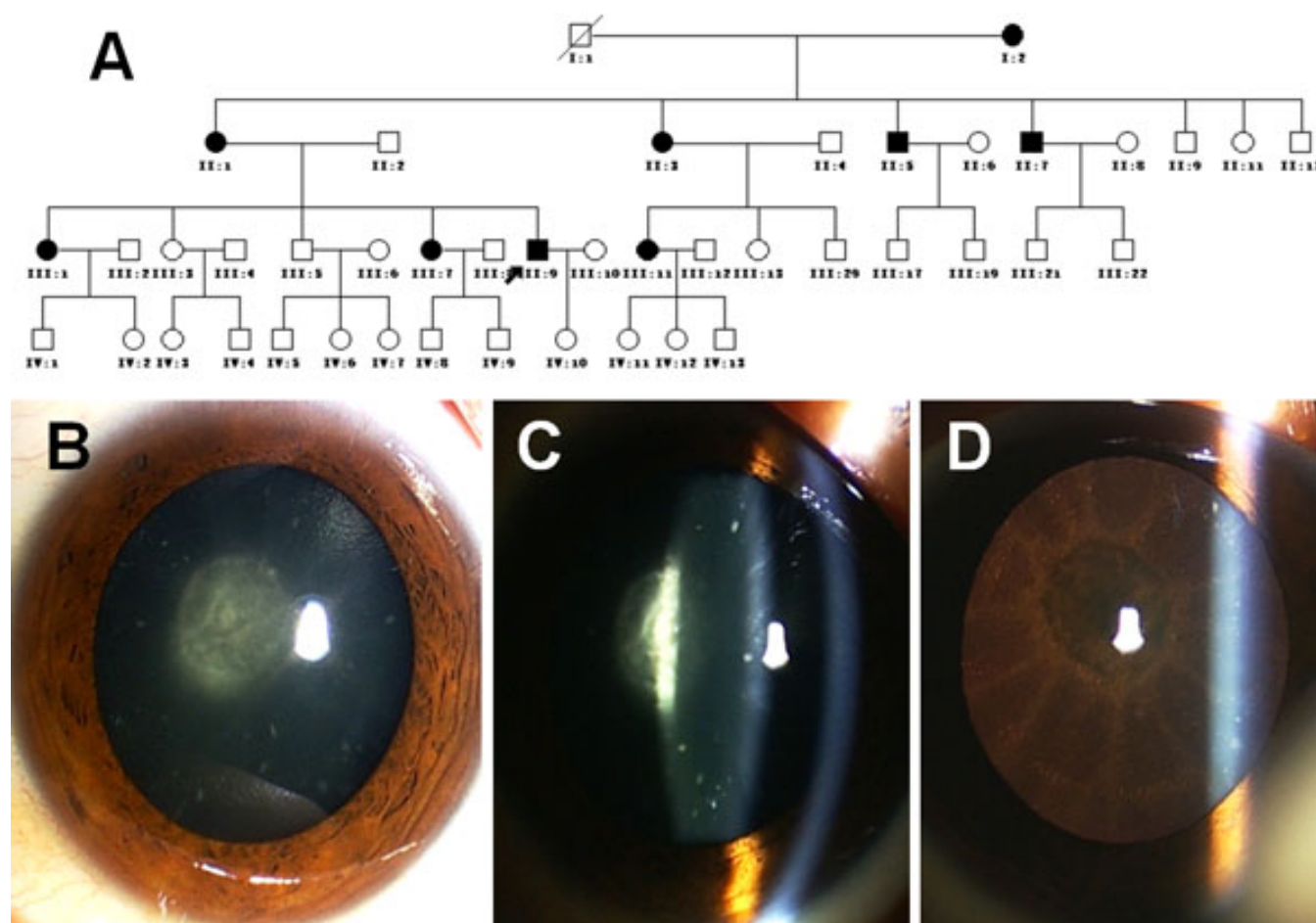


Figure 1. Clinical features of the family. **A**: Pedigree of the autosomal dominant congenital cataract. The proband is marked with an arrow. Squares and circles indicate males and females respectively. Black and white symbols represent affected and unaffected individuals, respectively. **B**, **C**, and **D**: Slit-lamp photograph of the proband (III:9) shows a posterior subcapsular cataract.

TABLE 1. POLYMERASE CHAIN REACTION PRIMERS AND PRODUCT SIZES OF HUMAN *CRYBB2* GENES.

Name	Primer sequence (5'-3')	Tm (°C)	Product size (bp)
CRYBB22F	5'-CCAGGTCCTCACTGCTGCTTC-3'	60.34	205
CRYBB22R	5'-CCCATTTTACAGAAGGGCAAC-3'	58.01	
CRYBB23F	5'-ACCCTTCAGCATCCTTTGG-3'	57.56	314
CRYBB23R	5'-GCAGACAGGAGCAAGGGTAG-3'	61.9	
CRYBB24F	5'-GCTTGGAGTGGAACTGACCTG-3'	61.92	244
CRYBB24R	5'-GGCAGAGAGAAAGTAGGATGATG-3'	61.98	
CRYBB25F	5'-GCCCCCTCACCCATACTC-3'	61.86	242
CRYBB25R	5'-CCCCAGAGTCTCAGTTTCTCG-3'	61.92	
CRYBB26F	5'CCTAGTGGCTTATGGATGCTC-3'	59.97	347
CRYBB26R	5'-TCTTCACTTGGAGGTCTGGAG-3'	59.97	

Eye Center of Affiliated Second Hospital, College of Medicine, Zhejiang University, Hangzhou, China. Informed consent in accordance with the Zhejiang Institutional Review Board was obtained from all participants and the study protocol adhered to the tenets of the Declaration of Helsinki. In total, 27 individuals participated: nine affected and 18 unaffected. Of the 18, nine were spouses (Figure 1A). Also, 100 unrelated control subjects were recruited. Detailed

medical histories were obtained by interviewing all individuals. All participants underwent ophthalmologic examinations, including visual acuity and slit-lamp examination with dilated pupils. Five of the affected members had undergone cataract extraction surgery. Phenotypes were documented by slit-lamp photography (Figure 1B-D). Blood samples were obtained by venipuncture, collected in Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ)

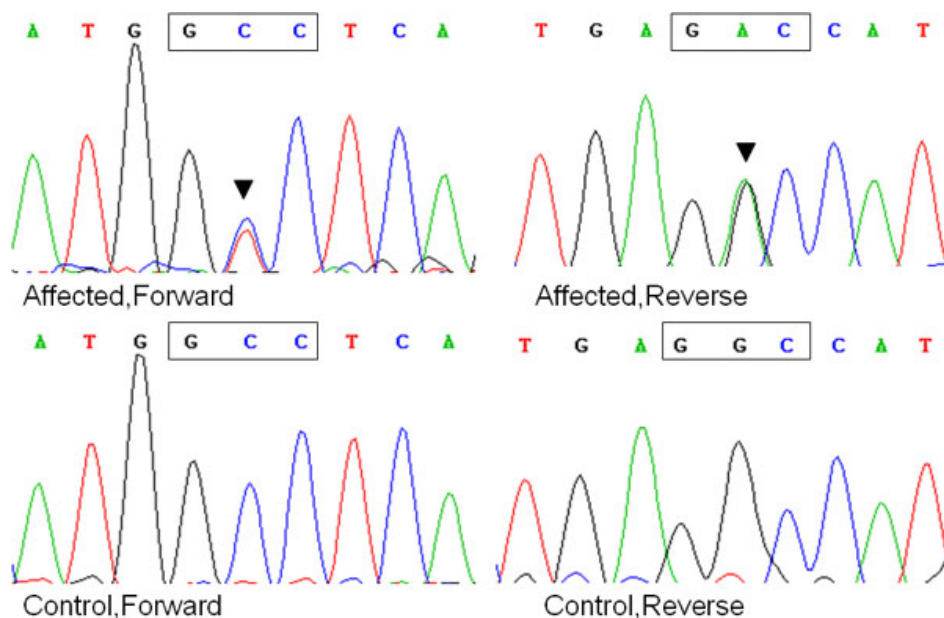


Figure 2. Forward and reverse sequence analysis of the affected and unaffected individuals in the ADCC Chinese family, showing a heterozygous mutation (c.5C>T) in exon 2 of *CRYBB2* (black triangles).

containing EDTA. Leukocyte genomic DNA was extracted using the QIAmp Blood kit (Qiagen, Duesseldorf, Germany).

Mutation analysis: Genomic DNA samples from affected and unaffected members of the family were screened for mutations in *CRYAA*, *CRYAB*, *CRYBA1*, *CRYBB2*, *CRYGC*, *CRYGD*, *GJA3*, *GJA8*, *PITX3*, and *CHMP4B* by a combination of direct sequencing and RFLP analysis. The coding regions of candidate genes were amplified by polymerase chain reaction (PCR) using previously published primer sequences (Table 1) [9,20-26]. The cycling conditions for PCR were 95 °C preactivation for 5 min, 10 cycles of touchdown PCR with 0.5 °C down per cycle from 60 °C to 55 °C, followed by 30 cycles with denaturation at 95 °C for 25 s, annealing at 55 °C for 25 s, and extension at 72 °C for 40 s. PCR products were isolated by electrophoresis on 3% agarose gels and sequenced using the BigDye Terminator Cycle sequencing kit V 3.1 (ABI Applied Biosystems; Sangon Co, China) on an ABI PRISM 3730 Sequence Analyzer (ABI), according to the manufacturer's instructions. Sequencing results were analyzed using Chromas 1.62 and compared with sequences from NCBI GenBank (*CRYAA*: 21q22.3; NM_000394, *CRYAB*: 11q22; NG_009824, *CRYBA1*: 17q11-q12; NM_005208, *CRYBB2*: 22q11.2; NM_000496, *CRYGC*: 2q33-q35; NM_020989, *CRYGD*: 2q33-q35; NM_006891.3, *GJA3*: 13q11-q13; NM_021954, *GJA8*: 1q21-q25; NM_005267, *PITX3*: 10q25; NM_005029, and *CHMP4B*: 20q11.2; NM_176812).

Restriction fragment length polymorphism analysis: Genetic variations were confirmed by the absence of cleavage sites for the restriction enzyme HaeIII in affected family members. For controls, we analyzed 100 DNA samples from ophthalmologically normal individuals of the same ethnic background as the family members. PCR products of exon 2

of the *CRYBB2* gene were digested for 10 h at 37 °C with HaeIII (TAKARA, Dalian, China), then electrophoresized in 5% polyacrylamide gels and analyzed under ultraviolet (UV) light.

Computational algorithms: Computational methods have been shown to be effective in predicting whether a specific amino acid substitution of a protein sequence is deleterious or neutral to the function of the protein. Three methods were used: Sorting Intolerant from Tolerant amino acid substitutions (SIFT), Polymorphism Phenotyping (PolyPhen), and Grantham score difference (Align-GVGD).

SIFT uses sequence homology to predict whether an amino acid substitution will affect protein function and, thus, potentially contribute to a disease. It is based on the approach that important amino acids tend to be highly conserved across species. SIFT, which assigns scores from 0 to 1, predicts substitutions with scores less than 0.05 as deleterious, whereas those greater than or equal to 0.05 are considered to be tolerated [27]. **PolyPhen** takes into account the evolutionary conservation of the amino acid subjected to the mutation and the physicochemical characteristics of the wild-type and mutated amino acid residue and the consequence of the amino acid change for the structural properties of the protein [6]. Grantham Variation (GV) measures the degree of biochemical variation among amino acids found at a given position in the multiple sequence alignment: Grantham Deviation (GD) reflects the 'biochemical distance' of the mutant amino acid from the observed amino acid at a particular position (given by GV). **Align-GVGD** can be used to predict the transactivation activity of each missense substitution [28]. A value of GV=0 corresponds to a residue that is invariant in the alignment, a value of GV of 60–65 is the upper limit of conservative variation across species, and a value of GV>100

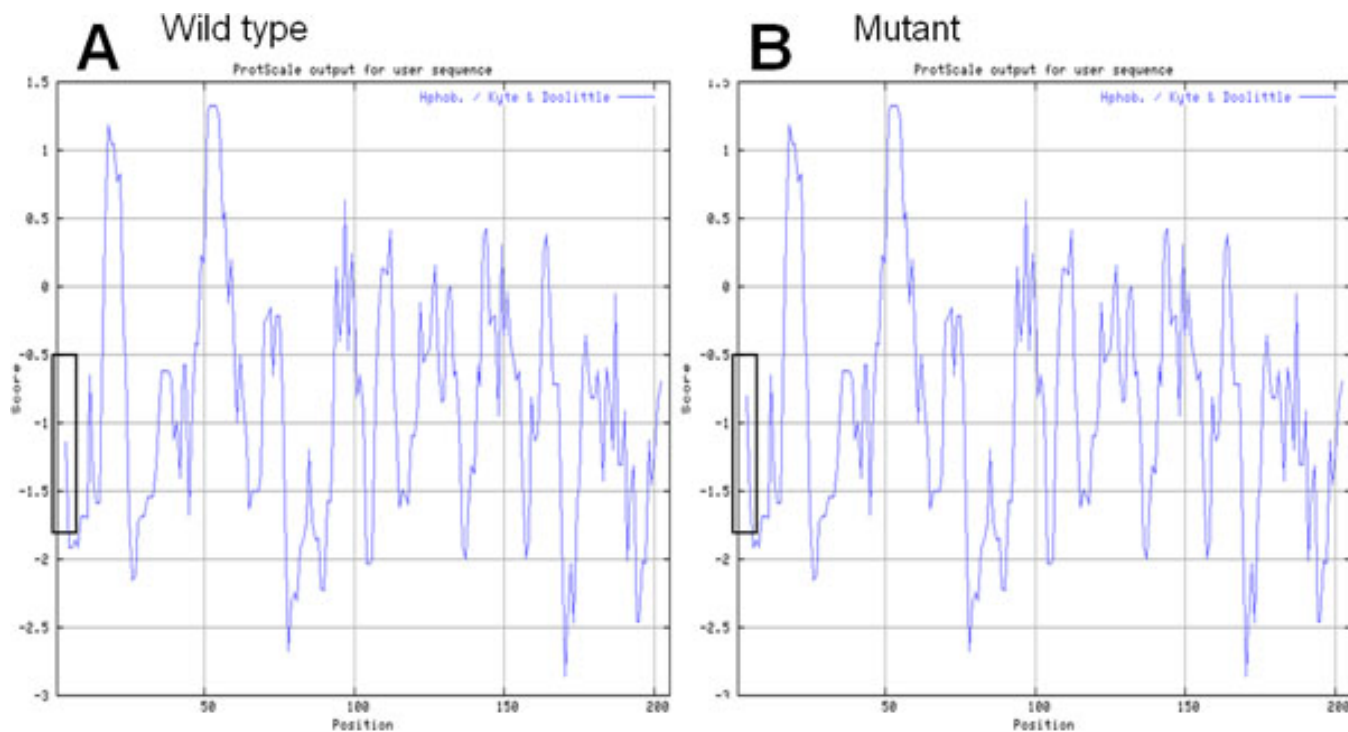


Figure 4. Hydropathy analysis of the mutant protein. Kyte-Doolittle hydropathy plot of CRYBB2WT (A) and CRYBB2A2V (B). The x-axis represents position of amino acids. The y-axis represents the hydropathy value in a default window size of 7. The region of interest is marked by white boxes. The increase in hydrophobicity in the mutant form is evident.

of the Chinese family under investigation, but was not detected in the 100 unrelated normal controls or unaffected pedigree members (Figure 3A).

Multiple-sequence alignment and mutation analysis: Using the NCBI websites, a multiple sequence alignment showed that the alanine at position 2 of human the CRYBB2 protein (*Homo sapiens*, NP_000487.1) is highly conserved in various species including *Oryctolagus cuniculus* (NP_001082786.1), *Bos taurus* (NP_777232.1), *Macaca mulatta* (NP_001116366.1), *Canis lupus familiaris* (NP_001041578.1), *Cavia porcellus* (NP_001166542.1), *Mus musculus* (NP_031799.1), *Xenopus laevis* (NP_001086491.1), *Gallus gallus* (NP_990506.2), and *Danio rerio* (NP_001018138.1; Figure 3B).

Computational analysis: Computational protein analysis of A2V CRYBB2 revealed the following results: the SIFT method revealed a score of 0.00, meaning the substitution is intolerant. PolyPhen analysis produced a score of 1.603, which is predicted to be “probably damaging.” Finally, Align-GVGD showed a score of GV0.00, GD65.28, which belongs to class C65 and means “most likely to interfere with function.” All of these results indicated the A2V substitution was likely deleterious and possibly contributed to the disease. The Kyte-Doolittle algorithm for hydrophobicity analysis showed the local hydrophobicity at and near the altered amino acid was increased (Figure 4).

DISCUSSION

Crystallins are known to constitute about 90% of the water-soluble proteins of the lens and contribute to transparency and refractive properties, due to a uniform concentration gradient in the lens. The vertebrate crystallins are divided into two families: α -crystallins and the β - and γ -crystallin families [30,31]. The β - and γ -crystallins share a common feature of anti-parallel β sheets in the proteins, referred to as the “Greek key motif.” The Greek key motif are agreed to be among the most stable of structures in proteins. Computer-based analyses suggest that they form an interdomain association: intramolecular in the γ -crystallins, and intermolecular in the β -crystallins. The detailed analysis of the mutations in the β - and γ -crystallin encoding genes might help to identify those amino acids which are the important corner stones for this function [32]. A second functional aspect of the Greek key motif is its Ca^{2+} binding properties. A human lens model of cortical cataract had been developed by Sanderson and his coworkers to study the role of Ca^{2+} in cataractogenesis [33]. Recently, Fischer et al. [34] reported on the effects of β - and γ -crystallins in axon regeneration of retinal ganglion cells.

The β -crystallin gene consists of six exons; the first exon is not translated, the second exon encodes the NH_2 -terminal extension, and the subsequent four exons are responsible for one Greek key motif each [35]. Mutations in the CRYBB2 gene in humans and mice have been reported to induce genetic cataracts [8,23,36-49] (Table 2). Previously, nine geographically distinct families have been reported with the

TABLE 2. PREVIOUS *CRYBB2* GENE MUTATIONS ASSOCIATED WITH CONGENITAL CATARACTS.

Bp exchange	Aa exchange	Biologic consequence	Origin of family	Reference
Human mutations				
c. G607A	p. V187M	Nuclear cataract	Basotho	[36]
c. C475T	p. Q155X	Sutural opacity and fish tail-like branches	American	[37]
c. C475T	p. Q155X	Cerulean	American	[8]
c. C475T	p. Q155X	ADCC	Canadian	[38]
c. C475T	p. Q155X	ADCC	Chilean	[39]
c. C475T	p. Q155X	Progressive polymorphic coronary ADCC	Indian	[40]
c. C92T	p. S31W	Coronary cataract	Chinese	[41]
c. C475T	p. Q155X	Cerulean ADCC	Chinese	[42]
c. C475T	p. Q155X	Progressive polymorphic	Chinese	[43]
c. G465T	p. W151C	Central nuclear	Indian	[44]
c. A383T	p. D128V	Dominant ring-shaped cortical cataract	Germany	[45]
c. C489A	p. Y159X	ADCC	Danish	[46]
c. C433T	p. R145W	ADCC	Danish	[46]
c. A440G	p. Q147R	ADCC	Danish	[46]
c. C449T	p. T150M	ADCC	Danish	[46]
Mouse mutations				
Intron 5: _57 A->T	Splicing: 19 new amino acids in front of exon 6	Progressive cataract		[47]
c. T560A	p. V187Q	Progressive cataract		[35]
585-587 Deletion	195-198 ΔQSVR	Progressive cataract		[48,49]

same nonsense mutation (Q155X) in exon 6 of the *CRYBB2* gene, which is associated with diverse phenotypes. This mutation creates a premature stop codon (Q155X) and results in an in-frame stop codon at nucleotide 475 of exon 6 that may cause a truncation of 50 amino acids from the COOH-terminus of β B2-crystallin, which destabilizes the domain structure of betaB2-crystallin. Using a mammalian two-hybrid system assay, spectroscopy (circular dichroism and fluorescence) and fast performance liquid chromatography (FPLC), the Q155X mutant shows not only decreased ordered structure and stability, but also a decrease in protein-protein interactions with β B2-crystallin mutant, which might contribute to cataract formation [50]. This mutation is explained by a gene conversion mechanism between the *CRYBB2* gene and its pseudogene *CRYBB2P1*. The diversity of the phenotypes may be caused by variations in the promoter region, possibly influencing the expression of the *CRYBB2* protein in the lens or other crystallin genes as modifiers from surrounding loci [37,39]. The other mutations were found in exons 3, 5, and 6. A2V in the *CRYBB2* gene characterized here is the first reported mutation in exon 2 (NH₂-terminal extension) of the *CRYBB2* gene.

β B2-crystallin, the major component of β -crystallin, is a homodimer at low concentrations, and can form a heterodimer with other beta-crystallins under physiologic conditions [51]. Based on the structure from X-ray refraction studies for homodimer β B2-crystallin, each subunit includes 16 β -

strands, eight in the NH₂-terminal domain and eight in the COOH-terminal domain [51]. The results of NMR spectroscopic studies indicate that the terminal extensions of beta B2-crystallin appear to be of little ordered conformation, are accessible to solvent and flex freely from the main body of the protein [52]. Earlier studies indicated that the NH₂-terminal extension of β -crystallin played an important role in oligomerization [53]. Recent experimental data suggest that the NH₂- and COOH-terminal arms appear to be involved in preventing the formation of higher homo- and hetero oligomers [54]. The long and flexible NH₂-terminal extension of the *CRYBB2* (PDB structure 1YTQ) might be critical for mediating protein interactions. Thus, the A2V substitution may influence homo- and heteromolecular interactions, which would contribute to cataract formation. In addition, lens crystallins are known to be susceptible to a wide variety of post-translational modifications such as acetylation, deamidation, methylation, oxidation, phosphorylation, and truncation of terminal extensions by thiol proteases. Age-related proteolytic processing of human lens β -crystallins occurs mainly at the NH₂-terminal extensions [55]. This single base substitution may play a role in post-translational modifications of crystallins, which would lead to protein structure and function alteration.

By the SIFT, PolyPhen, and Align-GVGD programs, we evaluated the possible effect of this amino acid substitution (p. A2V) on β B2-crystallin protein function. The programs

were used to determine whether a specific amino acid substitution would lead to an altered protein structure and function, based on sequence homology and structural information. As the isolated predictive value of these programs can be increased by their combination [6,56], it is believed that the A2V mutation alters the protein structure of the β B2-crystallin protein to such an extent it may contribute to the disease.

Also, hydropathy analysis revealed a variation in the physicochemical properties of the critical region in the A2V mutant (Figure 4B) compared with wild-type β B2-crystallin (Figure 4A). The environment surrounding the amino acid “V” in the mutant protein is more hydrophobic. Thus, the increase in hydrophobicity in the mutant form might affect the solubility of the mutant protein and hence contribute to cataract formation.

Conclusions: A novel A2V mutation of the CRYBB2 protein was identified and characterized in a Chinese family presenting with the posterior subcapsular type of congenital cataract. Further experiments on this cataract-related genetic defect and the factors that modify their variable phenotypes will improve our understanding of the mechanism of cataract formation and illuminate the developmental biology and biochemistry of the lens.

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