A novel γD-crystallin mutation causes mild changes in protein properties but leads to congenital coralliform cataract

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Purpose: To identify the genetic lesions for congenital coralliform cataract.

Methods: Two Chinese families with autosomal dominant coralliform cataract, 12 affected and 14 unaffected individuals, were recruited. Fifteen known genes associated with autosomal dominant congenital cataract were screened by two-point linkage analysis with gene based single nucleotide polymorphisms and microsatellite markers. Sequence variations were identified. Recombinant FLAG-tagged wild type or mutant γD-crystallin was expressed in human lens epithelial cells and COS-7 cells. Protein solubility and intracellular distribution were analyzed by western blotting and immunofluorescence, respectively.

Results: A novel heterozygous change, c.43C>A (R15S) of γD-crystallin (CRYGD) co-segregated with coralliform cataract in one family and a known substitution, c.70C>A (P24T), in the other family. Unaffected family members and 103 unrelated control subjects did not carry these mutations. Similar to the wild type protein, R15S γD-crystallin was detergent soluble and was located in the cytoplasm. ProtScale and ScanProsite analyses revealed raised local hydrophobicity and the creation of a hypothetical casein kinase II phosphorylation site.

Conclusions: A novel R15S mutation caused congenital coralliform cataract in a Chinese family. R15S possessed similar properties to the wild type γD-crystallin, but its predicted increase of hydrophobicity and putative phosphorylation site could lead to protein aggregation, subsequently causing opacification in lens.

Congenital cataract refers to lens opacification presented at birth or developed shortly thereafter. Its prevalence is up to 7.2 per 10,000 live births and renders about 10% of childhood blindness worldwide [1-7]. If left untreated, permanent visual loss usually occurs. Various etiological factors have been identified including infection, metabolic disorders, and genetic defects. About 18% of affected children have known family history of cataract [8]. The most common mode of genetic lesion is a single gene determinant in Mendelian inheritance. Autosomal dominant congenital cataract (ADCC) is a major form. Autosomal recessive and X-linked inheritance also exists [9]. According to the outward appearance, size, and location of lens opacity, congenital cataract (CC) is classified into various subtypes: whole lens, nuclear, lamellar, cortical, polar, sutural, pulverulent, cerulean, coralliform, and other minor subtypes [10,11]. The development of each type of cataract can be caused by distinctive etiological factors, especially defects in lens crystallins [9].

More than 20 genes out of 34 genetic loci mapped for isolated congenital cataract have been identified with specific mutations [12]. More than half of CC families carry mutations in 10 crystallin genes (namely crystallin alpha A (CRYAA), crystallin alpha B (CRYAB), crystallin beta B1 (CRYBB1), crystallin beta B2 (CRYBB2), crystallin beta B3 (CRYBB3), crystallin beta A1 (CRYBA1), crystallin beta A4 (CRYBA4), crystallin gamma C (CRYGC), crystallin gamma D (CRYGD) and crystallin gamma S (CRYGS)). About 25% of affected families have gene defects in membrane transport genes (major intrinsic protein of lens fiber (MIP), gap junction protein alpha 8 (GJA8), gap junction protein, alpha 3 (GJA3) and transmembrane protein 114 (TMEM114)) [13] and lens intrinsic membrane protein 2 (LIM2). The remaining are caused by mutations in genes encoding cytoskeletal proteins (beaded filament structural protein 1 (BFSP1)) [14] and beaded filament structural protein 2 (BFSP2)), transcription factors (paired-like homeodomain 3 (PITX3), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (MAF), and heat shock transcription factor 4 (HSF4)), chromatin modifying protein (CHMP4B) [15], and glucosaminyl transferase 2 (GCNT2) [16]. Forkhead box E3 (FOXE3), eyes absent homolog 1 (EYA1), and paired box gene 6 (PAX6) have been reported to cause congenital cataract in some patients associated with other anterior segment abnormalities [9]. The same mutation in different families or even within a family can result in drastically different morphologies and severity of lens opacification. On the other
hand, similar or identical cataract presentation may arise from mutations of different genes. These observations suggest that additional genes or modifying factors such as environmental regulators could play important roles in cataract onset, progression, and maturation.

CRYGD is a structural protein essential for lens transparency. Mutations of CRYGD are common genetic lesions causing different types of congenital cataracts. Among the reported families with congenital cataract caused by mutations of crystallin, one-third of them were associated with CRYGD. Until now, a total of 11 cataract-causing mutations (UniProt) have been reported including R15C, P24S, P24T, R37S, R59H, G61C, E107A, Y134X, W156X, G165fsX8, and R168W [17-21]. In this study, we added a novel R15S mutation to this list, affirming a causative role of CRYGD in coralliform type of congenital cataract.

METHODS

Patients and controls: This study adhered to the tenets of the Declaration of Helsinki and was approved by the ethics committees for medical research at The Chinese University of Hong Kong (Hong Kong, China) and Zhejiang University (Hangzhou, China). Two Chinese families with autosomal dominant congenital cataract were recruited at the University Eye Center (The Chinese University of Hong Kong; Family A; Figure 1) and the Department of Ophthalmology at the First Affiliated Hospital (College of Medicine at Zhejiang University; Family B; Figure 1). A total of 26 family members including 12 affected and 14 unaffected individuals attended this study, and informed consents were obtained from all participants. Unrelated Chinese control subjects (n=103) attending the hospital clinics for ophthalmic examinations were also recruited. They did not have any eye diseases except senile cataract and mild floaters. All subjects underwent complete ophthalmoscopic examinations. Family history and ophthalmic examination were documented by senior ophthalmologists. Peripheral venous blood was collected for genomic DNA extraction using QIAamp DNA kit (Qiagen, Valencia, CA).

Candidate gene screening: Fifteen candidate genes that account for most cases of ADCC were taken for linkage analysis (Table 1). A gene exclusion strategy was conducted by screening with single nucleotide polymorphisms (SNPs) and microsatellite markers. We obtained gene-based SNP markers in Han Chinese from HapMap with ABI SNP browser v.3.5 (Applied Biosystems, Foster, CA). TaqMan SNP genotyping assay and allelic discrimination was conducted on an ABI PRISM 7000 sequence detection system (Applied Biosystems). For genes without informative SNP markers or those that could not be excluded by SNP linkage analysis, microsatellite markers flanking to the target genes were chosen from the Marshfield genetic map. GeneScan was conducted on ABI Prism® 377 DNA sequencer. The pedigree and genotyping data were managed by GenoPedigree 1.0 and GeneBase 2.0.1. (Applied Biosystems). Two point LOD scores were calculated by the MLINK subprogram of FASTLINKAGE v.4.1P. A gene frequency of 0.0001 and penetrance of 100% were assumed for ADCC.

Mutation analysis: All coding exons and splice sites of CRYAA, CRYAB, CRYGC, CRYGD, and MAF were sequenced using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) and specific primers (Table 2) and detected by ABI Prism® 377 DNA sequencer. The data were compared with sequences from NCBI GenBank (CRYAA: NM_000394, CRYAB: NG_009824; CRYGC: NM_020989; CRYGD: NM_006891.3; MAF: NM_001031804).

Computational analysis: Effects of amino acid changes on the CRYGD protein structure, the isoelectric point (pI), and molecular weight (MW) were examined by Expasy proteomics. Local hydrophobicity was predicted by ProtScale. The protein sequence was scanned by ScanProsite to predict the effect of the mutation on specific motifs.
CRYGD expression, mutagenesis, and transfection: Human full-length wild type CRYGD was cloned to p3XFLAG-myc-CMV™-25 (Sigma, St Louis, MO) for epitope tagging to generate pFLAG/myc-CRYGDWT [19]. Missense mutations were introduced by a site-directed mutagenesis kit (Stratagene, La Jolla, CA) with specific oligonucleotides (Table 3). Correctness of the construct sequence was confirmed by direct sequencing. Preparation of pFLAG-CRYGD<sup>165fs</sup> was described as before [19]. Human lens B3 epithelial cells and COS-7 cells (ATCC, Manassas, VA) were maintained in Eagle’s minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotics [19]. Cells were seeded with 5×10<sup>5</sup> cells in a 60 mm (diameter) culture dish (Nunc,
Rochester, NY) overnight before transfection. Expression construct containing wild type or mutant CRYGD was mixed with FuGene HD reagent (Roche, Basel, Switzerland) at a ratio of 3 μl of FuGene per 1 μg of vector DNA in Opti-MEM® I (Invitrogen) supplemented with GlutaMAX™-I (Invitrogen) and incubated for 30 min. The mixture was then added to cells for up to 48 h.

Detergent solubility of wild type and mutant CRYGD: Cells transiently expressing wild type or mutant CRYGD were washed twice with ice-cold PBS and lysed in 2.5×10⁶ cells/ml lysis buffer, which contained 0.5% Triton X-100 (Tx; Sigma),

| Gene  | Amplicon | Primer sequences                                      |
|-------|----------|-------------------------------------------------------|
| CRYAA | 1        | 5'-CTCCAGGTCCCGGTGTA 5'-AGGAGAGGCGAGCACCAC            |
|       |          | 2 5'-CTGTCTCTGCAACCCAG 5'-CTGTCCCAACCTCTCAAGTGCC      |
|       |          | 3 5'-AATGATCCTCGGATTTTGGAG 5'-GGAAGCAAGAGGAGACAGACACC |
| CRYAB | 1        | 5'-TGTAGCTGCAAGCTGAGGAG 5'-TTCCAGTACAGGACTCTCCCG      |
|       |          | 2 5'-GAAGGATGAATTACCGGAACAG 5'-AGACATTTGTGAACCCCTGATC |
|       |          | 3 5'-GAGTTCAGGCGAGTGTAAT 5'-CTGCTGGGGAAACTTTCTTG      |
| CRYGC | 1        | 5'-TGCATAAAATCCCTACCGCC 5'-ACTCTGGGGCCATGATGG         |
|       |          | 2 5'-AGACTCATTTGGCTTTTCTTCCATCC 5'-GAATGACAGAAAGTCAATGC |
| CRYGD | 1        | 5'-CACGAGGCCCTCTGCTAT 5'-GCTTATGGGGAGCAACT            |
|       |          | 2 5'-CTTTTCTTCTTTTTTTTCTTGTGC 5'-GAAAGACACAAGACAAATGCTGCC |
| MAF   | 1        | 5'-CTCTGCAAGCCATCTGG 5'-CTGGTGCTGTTGTCTGATG          |
|       |          | 2 5'-GATCGCAACAGCCACCAG 5'-GAGAAGCGGTCGCTGAGTAG       |
|       |          | 3 5'-ACTTCGAGACCGCTTCTTCT 5'-TGGCGAGCATGGCTCTAG       |
|       |          | 4 5'-CCTTTACGCTGCGTTTGTAC 5'-AACCCCCAGGACAGAGGC       |

Five genes which were sequenced were listed. Forward and reverse primer sequences were provided for each amplicon of each gene.

| Mutations | Oligonucleotides with specific base change (underlined) |
|-----------|---------------------------------------------------------|
| R15C      | 5'-GACCGGGCCCTCCAGGGCTGCCACTATGAAATGCCAG              |
| R15S      | 5'-GACCGGGCCCTCCAGGGCTGCCACTATGAAATGCCAG              |
| P24T      | 5'-GAATGACAGACGGACCCACACCAAACTGCGACGACCCTAC           |
| G61C      | 5'-TACCTGCTGCGCCGCGTGCAGTACATGACC                     |

Constructs of four mutations in CRYGD were made. The sense oligonucleotides used in site-directed mutagenesis experiment were listed, respectively.

Table 2. Specific primers for direct sequencing.

Table 3. Sense oligonucleotides for site-directed mutagenesis in CRYGD.
for 2 min on ice [22]. After centrifugation, the Tx-soluble fraction was collected and denatured in SDS buffer containing 50 mM DTT. The pellet containing Tx-insoluble proteins was sonicated and denatured in SDS buffer containing 9 M urea. Both Tx-soluble and Tx-insoluble proteins equivalent to 7.5×10⁴ cells were analyzed by western blotting using monoclonal antibodies against FLAG, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or β-actin (Sigma).

Immunofluorescence staining: Cells grown on glass coverslips were fixed with freshly prepared neutral buffered 2% paraformaldehyde (Sigma) and permeabilized with 0.05% Tx [22] followed by incubations with mouse monoclonal antibodies against FLAG or mouse monoclonal antibodies against CRYGD (Abnova, Heidelberg, Germany). Rhodamine Red-X goat anti-mouse IgG (Invitrogen) was applied as the secondary antibody. The nuclei were contrast-stained with 4',6-diamidino-2-phenylindole (DAPI). The cells were examined under fluorescence microscopy (DMRB, Leica, Germany), which was equipped with Spot RT color system (Diagnostic Instruments Inc., Sterling Heights, MI).

RESULTS

Clinical investigations: Two pedigrees exhibited coralliform type of cataract in an autosomal dominant mode of inheritance. All affected patients had bilateral lens opacification, characterized by the appearance of white lines and processes extending from the nucleus to peripheral cortex, resembling the shape of sea coral (Figure 1). This was classified as the coralliform type of cataract by senior ophthalmologists in two eye centers (D.S.P.F., D.L., and J.P.T.). The lens opacity was less severe in terms of size and density in Family A than in Family B and did not result in significant loss of visual acuity (VA). In family A, except for one patient, II10, who had low vision due to high myopia, VA
of other patients ranged from 0.67 to 0.8 without lens surgery. Patient III-10 was recorded to have normal lens transparency at his first eye examination at the age of 2.5 years, but he was diagnosed to have cataract at 10 years old. Other family members were diagnosed after the age of five years. In Family B, all patients showed cataract within the first year after birth. The lens opacity caused obvious vision loss ranging from 0.04 to 0.7. Four of the six patients received cataract surgeries before the age of 30.

**Linkage analysis and DNA sequencing:** Through linkage analysis with selected SNPs and microsatellite markers (LOD score equal to minus infinity), 10 ADCC-associated genes were excluded. Five other genes were subject to direct sequencing. These genes were CRYAA (D21S1255; LOD score 0.3), CRYAB (D11S4151; LOD score 0.9), CRYGC, CRYGD (D2S2361; LOD score 1.51), and MAF (D16S503; LOD score 0.9) in Family A and CRYAB (D11S1793; LOD score 2.41), CRYGC, and CRYGD (D2S2208; LOD score 2.41) in Family B. Two sequence variants in exon 2 of CRYGD (NCBI accession number NM_006891.3) were identified to segregate with cataract in these two families. In Family A, a novel missense transversion, c.43C>A, which led to a substitution of arginine with serine at the 15th amino acid position (R15S), was detected (Figure 2). In Family B, a reported missense change, c.70C>A, which substituted proline with threonine at the 24th amino acid position (P24T), was found. All patients showed heterozygous changes. Neither the normal family members nor the 103 unrelated healthy controls carried these changes.

**Computational protein analysis of R15S CRYGD:** By Expasy proteomics, R15S CRYGD was predicted to have a reduced isoelectric point (pI) of 6.58 (compared to 7.0 for the wild type). The molecular weight (MW) was also slightly decreased (20.669 kDa for R15S CRYGD versus 20.738 kDa for wild type). By ProtScale analysis, the local hydrophobicity at and near the altered amino acid was increased (Figure 3). By ScanProsite, a hypothetical casein kinase II phosphorylation site was created due to the R15S mutation (Table 4).

**Cell specificity of R15S CRYGD solubility:** Recombinant FLAG/myc-tagged wild type or cataract-causing mutant CRYGD (R15S, R15C, P24T, G61C, and G165fsX8) was expressed in human lens epithelial B3 cells and COS-7 cells. Tx-soluble and Tx-insoluble fractions were western blotted for FLAG to detect wild type and mutant CRYGD proteins. The result of expression in lens B3 cells showed that except for G165fsX8, all known mutants of CRYGD remained Tx-soluble, which is similar to the wild type protein (Figure 4). The majority of G165fsX8 was present as Tx-insoluble. However, when expressed in COS-7 cells, an appreciable amount of mutant CRYGD protein became Tx-insoluble (Figure 4) and wild type CRYGD remained Tx-soluble. Band densitometry followed by normalization with housekeeping proteins (GAPDH for Tx-soluble and β-actin for Tx-insoluble fractions) revealed that about 8% of R15C and 17% of R15S CRYGD wereTx-insoluble (compared to 0.2% of wild type CRYGD). Similar observations were obtained in triplicate experiments.

**Cellular distribution:** In COS-7 cells, recombinant FLAG/myc-tagged R15S CRYGD was located predominantly in the cytoplasm and slightly in the nuclei (data not shown). No obvious inclusion was observed. This was similar to that observed in cells expressing wild type CRYGD.

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Figure 3. Hydrophobicity change of R15S CRYGD. The prediction by ProtScale analysis at Expasy indicated an increase of local hydrophobicity around the site of R15S mutation (Circle in panel B). A: The curve showed the hydrophobicity score of each amino acid of wildtype CRYGD. B: The curve was the hydrophobicity of R15S CRYGD. At the position of the 15th amino acid and its neighboring locations, the hydrophobicity scores increased which was indicated by the circle.
DISCUSSION

In this study, we identified a novel R15S mutation and a P24T change of CRYGD in two Chinese families showing ADCC of the coralliform type, which is a rare subtype of bilateral static congenital cataracts characterized by the appearance of opacity resembling the shape of sea coral. It shows white or cerulean opacificiation arranged in fusiform or spindle shape, extending from the center of the lens to the periphery but never reaching the capsule [10,11]. The irregular pattern of opacity running across the anatomic boundary of the cortical region indicates an altered arrangement of lens fibers, which associates with light scattering and reduction of transparency [11]. Until now, autosomal dominant transmission was the only mode of inheritance reported for coralliform cataract. Two loci, 2p24-pter and 2q33–35, have been mapped, and the specific gene, CRYGD (UniProt), in the latter locus was identified to associate with this cataract subtype [23]. Among all CRYGD mutations, R15C, P24T, and G61C were reported to be responsible for the coralliform phenotype [21,24-27]. In this study, the novel R15S change in CRYGD was found to cause congenital coralliform cataract. The index patient, III-10, in family A did not have a detectable lens opacity when he was 2.5 years old but was diagnosed with cataract at nine years of age. This was different from the effects of a previously reported R15C mutation of CRYGD, which caused the punctate type of congenital cataract at a much earlier age of disease onset [28]. Human CRYGD exists as a monomeric protein with a highly symmetric structure containing four Greek key motifs organized into two highly homologous β-sheet domains. The NH₂-terminal and COOH-terminal domains are covalently connected by a six-residue linker and interact non-covalently through the side chains of 10 amino acids across the domain interface. Due to these two conserved regions and a central hydrophobic domain interface, CRYGD exhibits high intrinsic stability [29,30]. For the R15C mutant, the additional reactive cysteine molecule at the protein surface could lead to the formation of disulfide cross-linkage, which in turn causes protein aggregation [31]. Nevertheless, substitution of the highly polar and charged arginine molecule

| Wild type | R15S |
|-----------|------|
| 5–8: TlyE | 5–8: TlyE |
| 35–37: SaR | 35–37: SaR |
| 75–77: SvR | 75–77: SvR |
| 78–80: ScR | 78–80: ScR |
| 87–89: ShR | 87–89: ShR |
| 166–168: SIR | 166–168: SIR |
| 50–53: NYSG | 50–53: NYSG |
| 60–62: RGD | 60–62: RGD |
| 71–76: GLsdSV | 71–76: GLsdSV |
| 158–163: GAtmAR | 158–163: GAtmAR |
| 91–98: Rly,EredY | 91–98: Rly,EredY |

All predicted functional sites in both wildtype and R15S CRYGD were listed in this table. The number is amino acid position. The asterisk indicates the newly formed casein kinase II phosphorylation site in R15S CRYGD.
by a less polar serine molecule as in R15S may cause lesser effects on protein conformation than the formation of an additional disulfide bond due to cysteine. Therefore, the R15S mutant is associated with the less severe late onset of cataract phenotype in our studied family. Although we detected no alteration of biochemical properties due to R15S in our cell expression studies, increased hydrophobicity at the R15S alteration of biochemical properties due to R15S in our cell phenotype in our studied family. Although we detected no mutant is associated with the less severe late onset of cataract by a less polar serine molecule as in R15S may cause lesser molecular Vision

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