A Novel Insertion Variant of CRYGD Is Associated with Congenital Nuclear Cataract in a Chinese Family

Xiaotong Zhuang1,2☯, Lianqing Wang3,4☯, Zixun Song1, Wei Xiao1✳

1 Department of Ophthalmology, Shengjing Hospital, China Medical University, Shenyang, China, 2 Department of Ophthalmology, The Fourth People’s Hospital of Shenyang, Shenyang, China, 3 Department of Medical Genetics, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, P. R. China, 4 Central Laboratory, Central Hospital of Zibo, Zibo, China

☯ These authors contributed equally to this work.
✳ xiaow@sj-hospital.org

Abstract

Objective
To investigate a novel insertion variant of CRYGD identified in a Chinese family with nuclear congenital cataract.

Methods
A Chinese family with congenital nuclear cataract was recruited for the mutational screening of candidate genes by direct sequencing. Recombinant N-terminal Myc tagged wildtype or mutant CRYGD was expressed in HEK293T cells. The expression pattern, protein solubility and subcellular distribution were analyzed by western blotting and immunofluorescence.

Principal Findings
A novel insertion variant, c.451_452insGACT, in CRYGD was identified in the patients. It causes a frameshift and a premature termination of the polypeptide to become Y151✳. A significantly reduced solubility was observed for this mutant. Unlike wildtype CRYGD, which existed mainly in the cytoplasm, Y151✳ was mis-located in the nucleus.

Conclusions
We have identified a novel mutation, c.451_452insGACT, in CRYGD, which is associated with nuclear cataract. This is the first insertion mutation of CRYGD found to cause autosomal dominant congenital cataract. The mutant protein, with loss of solubility and localization to the nucleus, is hypothesized to be the major cause of cataract in these patients.
Introduction

Congenital cataracts are a major cause of visual impairment and blindness in childhood [1]. The estimated prevalence is 1–6 per 10,000 live births, approximately one-third of which are believed to be inherited [2, 3]. Inherited cataracts are clinically highly heterogeneous, commonly exhibiting considerable inter- and intrafamilial phenotypic variation [4]. In non-consanguineous populations, inherited cataracts most frequently show an autosomal dominant pattern of inheritance, while autosomal recessive and X-linked patterns have also been reported [2, 5]. To date, more than 20 independent genes on different chromosomes have been shown to be associated with isolated congenital cataracts [6]. Among the congenital cataract families with causative mutations discovered so far, about half have mutations in crystallins, with the remainder divided among the genes for gap junction proteins, transcription factors, membrane transporter proteins, and cytoskeletal proteins [7].

Nuclear cataract, which was first described by Brown in 1924, is one of the most familiar types of severe congenital cataract [8, 9]. The visual acuity is remarkably reduced in patients with this kind of cataract because the opacity is located at the center of the lens [10]. Currently, autosomal dominant congenital nuclear cataracts have been linked to 11 specific causative genes [7], and most of the mutations are in the crystallin family of genes [9, 11–15].

In the present study, a four-generation Chinese family with congenital nuclear cataract was recruited. We identified a novel four-base insertion, namely c.451_452insGACT, in exon 3 of CRYGD after sequencing the candidate crystallin genes. This change led to a truncated protein Tyr151/C3. The mutant CRYGD exhibited reduced solubility, and localized incorrectly to the cell nucleus. To our knowledge, this is the first insertion mutation of CRYGD found to be disease-causing in congenital cataract.

Methods

Study samples

We collected blood samples from seven members (three affected and four unaffected) of a four-generation Han Chinese family with autosomal dominant nuclear cataract (Fig 1A) after obtaining informed consent and approval of the China Medical University Review Board. One hundred and three unrelated individuals were also recruited as study controls. Peripheral blood samples were collected for DNA analysis.

Mutation screening and sequence analysis

Genomic DNA was extracted from 500 μL of peripheral blood using a TIANamp Blood DNA Midi Kit (Tiangen, Beijing, China). After genomic polymerase chain reaction (PCR) performed, we sequenced the coding exons and their flanking intronic sequences of six crystallin family genes CRYAA, CRYBA1, CRYBB1, CRYBB2, CRYGC and CRYGD, for pathogenic mutations in the proband. The primers and reaction conditions used in PCR have been described in earlier reports [16–21]. To more clearly show the mutation c.451_452insGACT in CRYGD, the obtained fragment was cloned into pMD-18T vector (TaKaRa, Dalian, China) and sequenced. To confirm the mutation, we performed genomic polymerase chain reaction (PCR) using primers CRYGD forward (5’-TACGAGCTGTCCAACTACCGAG-3’ ) and reverse (5’-GAGAAATCTATGACTCTCCTCAG-3’ ) in the family and 103 unrelated individuals. The PCR fragments were separated by 8% polyacrylamide gel electrophoresis. The analysis of amino acid conservation around the mutation site was carried out by CLC DNA Workbench.
Genomic DNA fragments containing partial exon 2, intron 2 and partial exon 3 (the sequence before AAUAAA signal) were generated by PCR using primers CRYGD-2Fm (5′-ATCGGATCCGACTATGCCGACCACCAG-3′) and CRYGD-3Rm (5′-TGAGAATTCAGTTTCAAATTAAGAAACAAC-3′). The PCR fragments were cloned into the vector pcDNA3.1 (+) (Invitrogen, California, USA) at the BamHI and EcoRI sites. The constructs with the wildtype or mutant genomic inserts were verified by direct sequencing. The constructs were individually transfected into HEK293T or SRA 01/04 cells. For RT-PCR, total RNA was isolated using the Trizol reagent (Invitrogen) from the transfected cells and reverse transcription was performed using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa). PCR was then carried out with primers CRYGD-2FS (5′-GACTATGCGACCCACCAGCAG-3′) and pcDNA-R (5′-AACTAGAAGGCCAGTCAAGG-3′). The PCR products were sequenced using the primer CRYGD-2FS.
Bioinformatics analysis

The effect of the mutation c.451_452insGACT on splicing was predicted by Augustus (http://augustus.gobics.de/). The secondary structures of CRYGD were predicted by the GOR4 Secondary Structure Prediction Method. The protein physical and chemical parameters of the CRYGD protein were analyzed by ProtParam (http://web.expasy.org/protparam/). We also used the BEST/COREX server (http://best.bio.jhu.edu/BEST/index.php) to predict the stability of the wildtype and mutant proteins.

Plasmid constructs

The human CRYGD (GeneBank NM_006891.3) cDNA, encoding 174 amino acid residues, was obtained by PCR amplification from a Human Multiple Tissue cDNA Panel (Ovary tissue, BD, New Jersey, USA) and cloned into pCMV-Myc vector (Clontech, New Jersey, USA) at the site of EcoR I and Bgl II action. The primers used in the CRYGD construct were: sense 5’ – ATCCGAAATCCAATGAGATGACCCCTC – 3Y and antisense 5Y – GGATAGATCTCCAGGA GAAATCTAGCTAC – 3’ . The Y151 mutant was constructed by site-directed mutagenesis with the following primer: sense 5’ – GTCTGATGAGGAGACCTAGATGACCCCTC – 3’ ; antisense 5Y – GTAGCGCCTATAGTCAGTCCCCTGGCATCAGCAG – 3’ (the underline indicates the position of the base insertion). All the constructs were confirmed by direct sequencing.

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells and human lens epithelium lines SRA 01/04 (Cell Resource Center, IBMS, CAMS&PUMC) were routinely grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS at 37°C with 5% CO2. For transfection with plasmid DNA, the cells were cultured on six-well plates at 50% confluence, grown overnight, then transfected with the constructs using Lipofectamine 2000 (Invitrogen).

Protein over-expression analysis

After 36 h of transfection, the cultured HEK293T cells were harvested and lysed in RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS) (Beyotime, Shanghai, China) containing protease inhibitor cocktail (Roche, Basel, Switzerland) for 1h at 4°C. Centrifuge the samples at 12,000×g for 10 min at 4°C. The supernatant was collected and denatured in SDS loading buffer (TaKaRa). The precipitants containing insoluble proteins were washed three times with ice-cold PBS, sonicated, and denatured in SDS loading buffer containing 6 mol/L urea. After boiling, the protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to PVDF membranes (Millipore, Massachusetts, USA). The membranes were blocked with TBS buffer (pH 7.4) containing 0.05% Tween-20 and 5% nonfat milk. The blotted proteins were probed with anti-Myc antibody (9E10) (Santa Cruz Biotechnology, Santa Cruz, USA) or anti-β-actin antibody (ZSGB-Bio, Beijing, China), followed by incubation with Goat anti-Mouse IgG (H+L) (peroxidase conjugated) (Pierce, Illinois, USA). The blots were developed subsequently with an enhanced chemiluminescence (ECL) system (Pierce).

Immunofluorescence

HEK293T cells grown on glass coverslips were fixed with 4% paraformaldehyde (Sigma Aldrich, Shanghai, China), permeabilized with 0.05% Triton-X 100, and blocked with 5% bovine serum albumin (BSA). Anti-Myc antibody (9E10) incubations were carried out in 5% BSA at a dilution of 1:200. After incubation, the cells were washed with PBS three times. The
secondary antibody conjugated DyLight 488 (EarthOx, California, USA) (1:800) was added; this had been tested and no cross-reactivity found. The nucleus was stained with 500 ng/ml DAPI (Sigma Aldrich). Following three final washes with PBS, coverslips were mounted in antifade reagent (Beyotime). The samples were examined by fluorescence microscopy equipped with a DP controller system (DP70, Olympus, Japan).

**Results**

**Clinical features**

We ascertained a four-generation Chinese family with autosomal dominant nuclear congenital cataracts (Fig 1A). All affected individuals showed nuclear cataract, which presented several months after birth. The visual acuity was impaired gradually as the lens opacity increased, and was seriously reduced at 12–13 years old. We failed to get the visual acuity of proband because she is in early childhood (S1 Table). The affected individuals showed an opacity in the central nucleus region of both lenses. The Y-sutures were also involved with prominent opacity (Fig 1B). No systemic disease or other ocular disease was found.

**Mutation confirmation in CRYGD**

Mutation screenings were performed for all six candidate crystallin genes, and a heterozygous variant, c.451_452insGACT, was identified in exon 3 of CRYGD (Fig 2A). The variant led to the substitution of a newly formed stop codon for a phylogenetically conserved tyrosine residue (p.Tyr151*) (Fig 2B), and was only identified in the affected individuals. With the exception of several nonpathogenic SNPs, no other variants were detected. On polyacrylamide gel electrophoresis, the variant was confirmed in all affected individuals but was not detected in unaffected family members or 103 unrelated Chinese controls (Fig 2C).

**The mutation has no effect on splicing**

To investigate whether c.451_452insGACT has effect on splicing, it was predicted by Augustus. It showed the splicing was not affected by the mutation (data not shown). To confirm the result, minigene assay was performed. Using primers from exon 2 and pcDNA 3.1, PCR was carried out to analyze the transcription fragments. There were no obvious difference in the length between wildtype and mutant (Figure B in S1 File). Consistent with the results of bioinformatics analysis, splicing was not affected by the mutation c.451_452insGACT, which was confirmed by direct sequencing (Figure C in S1 File).

**Bioinformatics analysis of Y151* on protein level**

The mutant protein was truncated by 24 amino acids at the C-terminus when compared with the wildtype, and the fourth Greek key motif (amino acid 129–171) was partially absent. Both the extended strand and the random coil were reduced, resulting in destruction of the secondary structure (Fig 3A). The truncated CRYGD had an acidic isoelectric point (PI), which may be involved in protein aggregation. The mutation rendered CRYGD more unstable and decreased its solubility (Table 1). Moreover, the stability constant (Kf) at the per residue level showed that the mutant CRYGD favored the unfolded states at the C-terminus (Fig 3B).

**The mutant CRYGD has decreased solubility**

Myc-tagged wildtype and Y151* CRYGD constructs were transiently expressed in HEK293T cells and were detected using anti-Myc antibody (9E10). Western blotting showed that the molecular weight of recombinant mutant CRYGD had decreased as a result of the loss of 24
amino acids (Fig 4A, upper). Moreover, the mutant CRYGD had a greatly reduced level of protein level in the supernatant when compared with wildtype (Fig 4A, upper; Fig 4B). The mutant protein aggregated mainly in the precipitant where the wildtype was not detected (Fig 4A, lower). The results indicated that the mutant protein possessed decreased solubility.

Mis-localization of mutant CRYGD to the cell nucleus
To confirm the pathogenicity of the mutation Y151/C3, immunofluorescence assay was performed to detect the subcellular localization of mutant CRYGD. Myc-tagged wildtype CRYGD was localized in both cytoplasmic and membrane regions, whereas Y151/C3 was redistributed in the nucleus as a spot-shaped structure (Fig 5). Minimal staining in the cytoplasm was also observed. The aggregation of the mutant protein in the nucleus showed that it failed to form crystallin and perform its intrinsic function.

Discussion
In this study, we identified a novel deletion mutation (c.451_452insGACT) in CRYGD, which caused a truncated polypeptide (p.Tyr151*). Our expression studies demonstrated that the mutant was highly insoluble and had an abnormal location. The mutant CRYGD may affect lens fiber differentiation thus interfering with lens transparency [22].

Lens transparency is maintained mainly by structural crystallins. Crystallins consist of a core set of three protein families, the α-, β- and γ-crystallins, which are defined by the sizes of the oligomers that they form [23]. Alteration of both the function and the molecular properties of crystallins may cause the breakdown of lens microstructure, which results in changes in the refractive index and increased light scattering [22]. γD-crystallin, encoded by CRYGD, is one of
The important crystallins. An impairment in γD-crystallin expression may be responsible for cataract [3, 22].

γD-crystallin is composed of two individual domains that each consist of two Greek key motifs (GKM) [3]. Each GKM is composed of four antiparallel β-strands. Up to 2015, a total of 19 mutations in CRYGD were reported responsible for cataracts, most of which are missense mutations. However, little functional importance of the mutants was illustrated in the previous reports (S2 Table.). All the mutations distribute in the four GKM. Intriguingly, the truncated mutations are mainly in the GKM4 of the C-terminus (5/7) (S1 Fig). However, the nonsense mutation c.51T>G (Y17*) and c.168C>G (Y56*) Also, the Y151* mutation is located in the same domain. The insertion c.451_452insGACT in exon 3 of CRYGD is predicted to cause a mutation with a significant impact on the secondary structure and stability of the protein.

**Table 1. Comparison of CRYGD wildtype and mutant.**

| Protein characteristics | WT  | Y151* |
|------------------------|-----|-------|
| Number of amino acids  | 174 | 150   |
| Theoretical PI         | 7.00| 6.05  |
| Instability index      | 54.89| 56.82 |
| Aliphatic index        | 63.85| 63.67 |
| Grand average of hydropathicity | -0.804 | -0.799 |

The physical and chemical parameters of the protein were analyzed by ProtParam.
Fig 4. Western blot analysis of CRYGD over-expression in HEK293T cells. (A) The truncated CRYGD showed decreased solubility. In the supernatant, the mutant protein was truncated and was present in much lower amounts than the wildtype. In the precipitant, there was more mutant protein and the wildtype was not detected. (B) Quantification by band densitometry indicated the prominent reduction of mutant CRYGD in the supernatant (p<0.05).

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Fig 5. Localization of Myc-tagged wildtype or Y151* CRYGD in HEK293T cells. Immunofluorescence of Myc (green fluorescence) showed the distribution of wildtype CRYGD in both cytomembrane and cytoplasm. However, the mutant CRYGD was mainly localized in the nucleus, in the form of granular deposits. Scale bar: 10 μm.

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premature stop codon, which leads to the deletion of the C-terminal residues 151–174 (Fig 3A). As shown in the diagram (Fig 3B), the truncated protein destabilizes the C-terminal domain. Moreover, the aggregation state of the mutant γD-crystallin was also affected (Fig 5). Several usually buried residues of the mutant γD-crystallin may be exposed on the surface, which means that the protein displays lower solubility and structural stability [24]. The loss of the C-terminal tail in γD-crystallin leads to substantial intermolecular aggregation, which generates light-scattering particles [25].

It is unclear why the aggregated mutant γD-crystallin was located in the nucleus. There are no amino acid residues related to nuclear localization in the lost C-terminal tail. Asp156, Arg163 and Arg168, the charged residues of wildtype γD-crystallin, are involed in charge interactions [26]. They may play an important role in the processing and subcellular localization of the protein [27]. It will be intriguing to explore how the incorrect localization may have resulted from alteration of the physicochemical properties of the protein.

In summary, we have identified c.451_452insGACT as a novel causative mutation in CRYGD responsible for autosomal dominant congenital nuclear cataract. Our results provide a novel insight into the molecular mechanisms involving CRYGD that underlie the pathogenesis of human congenital cataract.

Supporting Information

S1 Fig. The distribution of mutations in CRYGD responsible for cataracts. (TIF)

S1 File. The effect of the mutation c.451_452insGACT on splicing. The schematic diagram of DNA fragments used in the constructs (Figure A). Agarose gel electrophoresis showing no difference in the PCR fragments between wildtype and mutant (Figure B). Sequence chromatogram showing the c.451_452insGACT mutation has no effect on splicing (Figure C). (TIF)

S1 Table. Clinical information of the family members. (PDF)

S2 Table. γD-crystallin mutations, related phenotypes and functional changes. (PDF)

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Author Contributions

Conceived and designed the experiments: WX. Performed the experiments: XTZ LQW ZXS. Analyzed the data: WX ZXS. Contributed reagents/materials/analysis tools: WX. Wrote the paper: XTZ LQW.

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