• Basic Research •

A novel frameshift mutation in CX46 associated with hereditary dominant cataracts in a Chinese family

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Abstract

• AIM: To investigate the genetic mutations that are associated the hereditary autosomal dominant cataract in a Chinese family.

• METHODS: A Chinese family consisting of 20 cataract patients (including 9 male and 11 female) and 2 unaffected individuals from 5 generations were diagnosed to be a typical autosomal dominant cataract pedigree. Genomic DNA samples were extracted from the peripheral blood cells of the participants in this pedigree. Exon sequence was used for genetic mutation screening. In silico analysis was used to study the structure characteristics of connexin 46 (CX46) mutant. Immunoblotting was conduceted for testing the expression of CX46.

• RESULTS: To determine the involved genetic mutations, 11 well-known cataract-associated genes (cryaa, cryab, crybb1, crybb2, crygc, crygd, Gja3, Gja8, Hsf4, Mip and Pitx3) were chosen for genetic mutation test by using exon sequencing. A novel cytosine insertion at position 1195 of CX46 cDNA (c.1194_1195ins C) was found in the samples of 5 tested cataract patients but not in the unaffected 2 individuals nor in normal controls, which resulted in 30 amino acids more extension in CX46C-terminus (cx46fs400) compared with the wild-type CX46. In silico protein structure analysis indicated that the mutant showed distinctive hydrophobicity and protein secondary structure compared with the wild-type CX46. The immunoblot results revealed that CX46 protein, which expressed in the aging cataract lens tissues, was absence in the proband lens. In contrast, CX50, alpha A-crystallin and alphaB-crystallin expressed equally in both proband and aging cataract tissues. Those results revealed that the cx46fs400 mutation could impair CX46 protein expression.

• CONCLUSION: The insertion of cytosine at position 1195 of CX46 cDNA is a novel mutation site that is associated with the autosomal dominant cataracts in this Chinese family. The C-terminal frameshift mutation is involved in regulating CX46 protein expression.

• KEYWORDS: connexin 46; cataract; congenital; ocular lens; autosome dominant heredity; genetic mutation

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INTRODUCTION

Congenital cataracts, which affect about 0.015%-0.03% of children vision in the world, are the main cause of childhood blindness. The hereditary autosomal dominant and recessive cataracts account for one third of congenital cataracts[1]. Such type of cataracts, which usually develops in infants with abnormal eye development, is closely associated with autosome genetic mutation (*e.g.* genetic mutations in crystallins, gap-junction proteins, lens structural intermediate filaments and transcription factors)[2]. These mutations impair the lens development at different developmental stages.

Gap junctions are channels formed by two hemichannels (connexons) that mediate the cell-to-cell communication of ions, ATP, peptides and other nutrients. Each hemichannel is composed of six protein subunits termed connexins (CXs). About 20 CXs have been identified in mammalian tissues, and they usually form three types of gap junctions: 1) homotypic channels with two identical connexons composed of one type of CX subunit; 2) heterotypic channels with homomeric connexons, each containing a different type of CX; and 3) heterotypic channels with heteromeric connexons[3-4]. In lens,

gap junctions are composed of three types of CXs: CX43 (or Gja1) is present in lens epithelial cells; CX46 (or Gja3) mainly expresses in the fiber cells; and CX50 (or Gja8) is present in epithelial and fiber cells[5-7]. The CX23 protein is also expressed in embryonic lens fiber cells, but it is unclear whether this CX can form gap junctions[8]. Knocking out CX46 or CX50 genes cause the microphthalmia and congenital cataracts in mouse models[9-10]. Genetic mutations in CX46 or CX50 genes are closely associated multiple hereditary diseases such as cataracts and neurodegenerative diseases.

CX46 protein, which contains 435 amino acids, has four transmembrane domains and is predominantly expressed in mature fiber cells. The 23 mutations in CX46 protein were identified to be closely associated with hereditary autosomal cataracts[11-13]. Most of these mutations occur in the N-terminal, first transmembrane and extracellular domains. One frameshift mutation, which is at fs380 of CX46, was reported to be associated with hereditary autosomal dominant cataracts in a Chinese family[14]. The congenital cataracts linked to the CX46 mutations are heterogeneous in age of onset and cataract appearance, including nuclear, total, posterior polar, coppock-like and zonular pulverulent types. Hemichannel dysfunction and/or endoplasmic reticulum-associated degradation (ERAD) induced by misfolded CX46 mutants in the endoplasmic reticulum were found to be the common molecular mechanisms responsible for the CX46-associated cataracts[15].

In the present study, we looked at the genetic mutations that are possibly associated with an autosomal dominant cataract pedigree consisting of 5 generations and 27 individuals. Using direct exon sequencing, we found a novel insertion of cytosine at site 1195 in the CX46 cDNA. This insertion causes a frameshift mutation in the C-terminus of CX46, resulting in CX46 protein dysfunction, which should be closely associated with this cataract pedigree.

MATERIALS AND METHODS

Subjects and DNA Preparation A Chinese cataract pedigree, which contains 27 patients involving five generations, presented in eye clinic in Kaifeng Eye Hospital. To do the genomic mutation analysis, the consents were noticed to all the participants. For genomic DNA extraction, the peripheral blood samples were collected from 5 affected and 2 unaffected individuals in this pedigree, and 100 normal volunteer students from Henan University School of Medicine. The genomic DNA was isolated according to the standard protocol in the blood genomic DNA isolation kit (DP348, TIANGEN Biotech, Beijing, China) for exon sequencing. All experimental procedures described in this study were approved by the Ethics Committees of both Henan University and Kaifeng Centre Hospital.

Mutation Screening For exon sequencing, 11 candidate genes (cryaa, cryab, crybb1, crybb2, crygc, crygd, Gja3, Gja8, Hsf4, Mip and Pitx3) that are known to be associated with hereditary autosomal dominant cataracts were chosen for exon sequencing. All exons and exon-intron junctions of these 11 candidates were amplified with rTaq DNA polymerase (Takara, Japan) and their respective primers. The PCR products were validated by DNA sequencing (GENEWIZ, Suzhou, China). For exon sequencing of CX46, the primer set 1: CX46-1 (forward: 5' cccatcagccccatcccagta 3' and reverse: 5' ggcgggggatcggctgtc 3') was used to amplify coding region of exon 2 , while primer set 2: CX46-2 (forward: 5' aggcccacggagaagaccatc 3' and reverse: 5' gcattgaacacggaaacctgatct 3') was for amplifying the genomic region of exon 2-intron junction. Both primer sets produced a unique PCR band in 1% argrose gels (data not shown).

Immunoblotting Assay The lens anterior epithelia and fiber tissues were collected from proband and other unrelated age-matched cataract patients during the intraocular lens implantation surgery. The tissues were lysed with NP-40 lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40) containing the protease inhibitor cocktail (Roche). Equal amounts of protein samples were separated with SDS-PAGE and transferred onto PVDF membranes. After blocking in 5% fat-free milk, the membranes were incubated with the primary antibodies overnight at 4℃. After being washed in PBST buffer, the membranes were incubated with the secondary antibodies conjugated with hydrogen-peroxide (HRP) (Thermo Fisher, USA) and developed in enhanced chemiluminescence (ECL) buffer. The signals were detected by exposing the membranes to X-ray film. Antibodies against GAPDH (HRP-60004) and CX46 (22717-1-AP) were purchased from Protein tech (Wuhan, China). Alpha A-crystallin antibodies (NBP2-12875) were from Novus (Littleton, USA), alpha B-crystallin antibody (sc-22744) from Santa Cruz (Shanghai, China), and CX50 antibody (SAB1300562) from Sigma (St. Louis, USA).

RESULTS

Clinical Examinations and Pedigree Analysis The proband presented to the eye clinic with nuclear cataracts. According to the traits, a pedigree was created to represent the status of cataracts in each family member (Figure 1A and Table 1). The proband's great-grandparents (I-1 and I-2) died without clearly diagnostic cataracts. However, generation I had three offspring (generation II). One male (II-1) and one female (II-3) are affected individuals while male II-2 is unaffected one. Female II-3 had 6 offspring. Two males (III-2, III-4) and 3 females (III-6, III-10 and III-11) are affected individuals, while one female III-8 is unaffected. The offspring of male III-2, III-4 and female III-6, III-10 are all affected (IV-1 to IV-8, IV-12 and IV-13). IV-12 is the proband. Female III-11 has two offspring, one is the unaffected male (IV-15), the other is the affected female (IV-16). IV-12 (proband) has two unaffected offspring (male

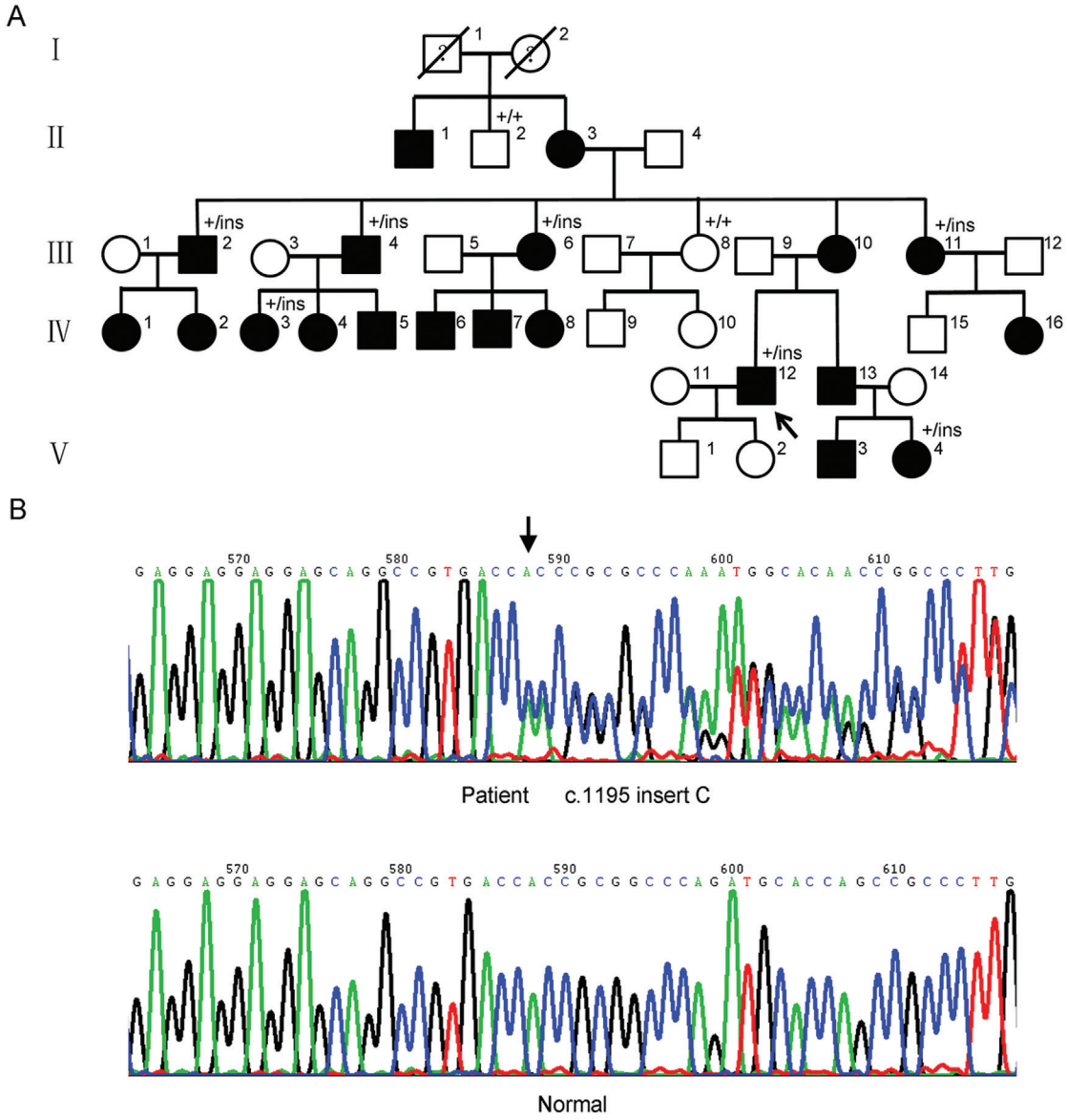

Figure 1 A novel cx46fs400 frameshift mutation is associated with this Chinese autosomal dominant cataract pedigree A: Pedigree of a Chinese family affected with congenital cataracts. The empty symbols represent the unaffected members and filled symbols represent the affected members. Symbol with a question marker indicate members with unknown phenotype. The proband is indicated with an arrow. There is no consanguinity in this pedigree. +/+: Wild-type; +/ins: Insert mutation. B: DNA sequencing profile of a novel cytosine insertion site around position c.1195 from the proband (IV-12) and unaffected individual (III-8).

V-1 and female V-2), while IV-13 has two affected offspring (male V-3 and female V-4). These results suggested that this pedigree was regulated by the autosomal dominant trait. We collected the blood samples from 7 participants in this family for DNA extraction. Five affected individuals [including proband (IV-12), III-2, III-4, III-6, and V-4] and 2 unaffected individuals (III-8, II-2) were subjected to the exon sequencing.

Identification of a Novel CX46 Frameshift Mutation (cx46fs400) in the Family To determine the genetic mutations that were associated with this cataract pedigree, the DNA samples from 5 cataract patients and 2 unaffected members were subjected to the exon sequencing. Eleven candidate genes (cryaa, cryab, crybb1, crybb2, crygc, crygd, CX46, CX50, Hsf4, Mip and Pitx3), in which the mutations are known to be associated a variety of autosomal dominant cataracts, were chosen as screening targets in this study. The sequencing results indicated that in addition to four SNPs (rs11603779, rs2330991, rs2330992 and rs4049504) in cryab and crybb2, a cytosine insertion at position 1195 of the CX46 cDNA (c.1194_1195ins C) was observed in all tested cataract patients but not in the unaffected family members *e.g.* III-8 and II-2 (Figure 1B), nor in the 100 normal control DNA samples (data not shown).

Structural Property of the CX46 Mutant by in Silico Analysis CX46 protein consists of 435 amino acids. This cytosine insert caused amino acid frameshift in the CX46 protein at threonine 400 (cx46fs400) resulting in 31 more amino acids added to CX46's C-terminal tail compared to the

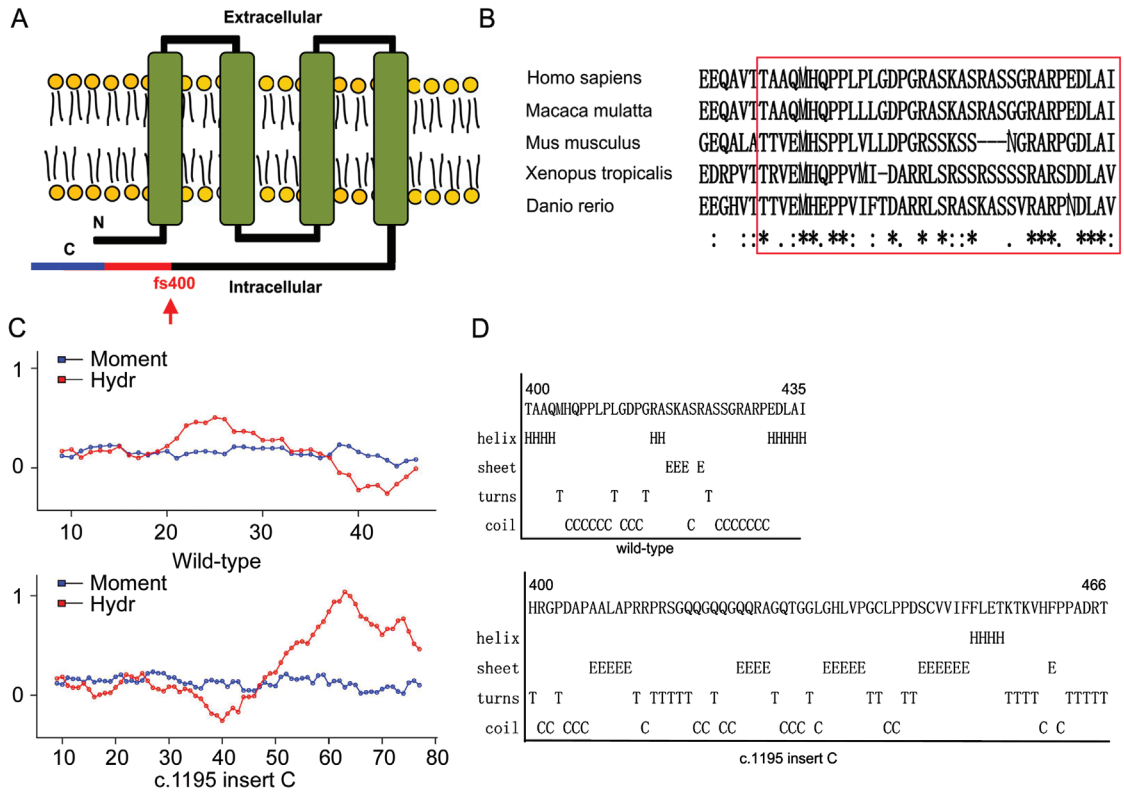

Figure 2 Characterization of cx46fs400 frameshift mutant A: A membrane topology diagram illustrating the location of cx46fs400 at the C-terminus of CX46. The substituted 35 animo acids are colored in red, the blue represents the 31 more amino acids extension. N: N-terminus; C: C-terminus. B: Alignment of the amino acid sequences of the wild type CX46 C-terminal tail among different species. Asterisks indicate the highly conserved residues. C: Hydrophobicity analysis of the C-terminal tail of CX46 frameshifted mutant *vs* wild type. The red line represents the hydrophobicity. D: Predicted secondary protein structure of CX46 C-terminal tail of frameshift mutant *vs* wild type. H: Alpha helix; E: Beta sheet; T: Turn; C: Coil.

Table 1 The clinical features of family participants in the present study

ID	Age (a)	Sex	Disease status	Onset age (a)	Surgery age (a)
Ⅲ 2	67	M	Aff	<10	65
Ⅲ 4	63	M	Aff	<10	62
Ⅲ 6	62	F	Aff	<10	60
Ⅲ 8	59	F	Un	-	-
Ⅲ 10	56	F	Aff	<10	50
Ⅲ 11	54	F	Aff	<10	51
Ⅳ 1	42	F	Aff	4	15
Ⅳ 2	41	F	Aff	3	14
Ⅳ 3	41	F	Aff	3	15
Ⅳ 4	39	F	Aff	5	13
Ⅳ 5	36	M	Aff	3	10
Ⅳ 6	35	M	Aff	7	7
Ⅳ 7	33	M	Aff	5	5
Ⅳ 8	32	F	Aff	4	4
Ⅳ 12	31	M	Aff	3	30
Ⅳ 13	29	M	Aff	4	22
Ⅳ 16	25	F	Aff	10	10
Ⅴ 3	5	M	Aff	3	3

M: Male; F: Female; Aff: Affected; Un: Unaffected.

parental one (Figure 2A, 2D). To characterize the affected amino acids at the C-terminus, we blasted the wild type C-terminal amino acids of human to that of macaque, mouse, xenopus and zebrafish, and found that the amino acids in the C-terminus of CX46 were highly conserved among the species (Figure 2B). However, the frameshift mutation completely changed the homology. Hydrophobicity analysis using the Heli-Quest online tool suggested that the extra amino acids derived from frameshift were more hydrophobic than wild type (Figure 2C). Analysis of the protein secondary structure using the Garnier Peptide Structure Tool indicated that the CX46 mutant contained more β-sheets and turns than the wild-type protein. In contrast, the C-terminus of wild-type CX46 contained more helix-coil motifs compared to the mutant (Figure 2D). These data suggested that this frameshift mutant completely changed the biophysiological characteristics of CX46 intracellular domain.

The Novel cx46fs400 Mutation Impairs CX46 Protein Expression in Lens Tissues To study whether cx46fs400 mutation affects CX46 protein expression, the expression level of the CX46 mutant protein in the proband's lens tissue were immunoblotted. Lens tissues from two individuals with canonical age-matched cataracts were used as controls. As

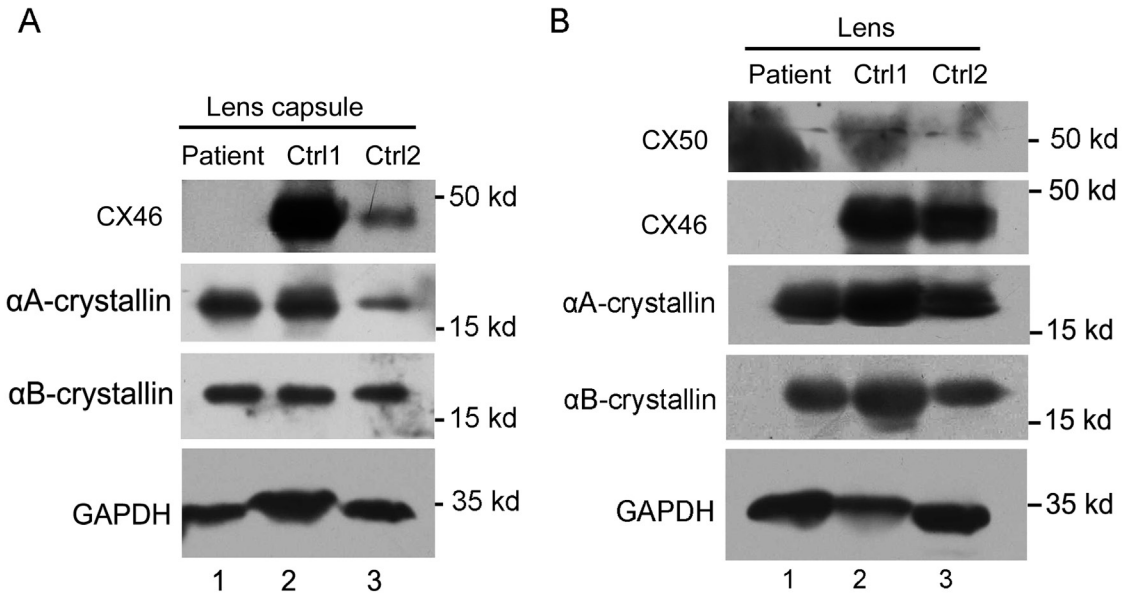

Figure 3 Cx46fs400 frameshift mutant impairs CX46 protein expression in the proband's cataract lens tissue A: The amount of CX46, alpha A-crystallin and alpha B-crystallin protein in lens anterior epithelia from proband (lane 1) and other age-matched cataracts (lanes 2 and 3) were analyzed by immunoblot. B: Immunoblotting the protein expression of CX46, CX50, alpha A-crystallin and alpha B-crystallin in lens fiber tissues of proband (lane 1) and age-matched cataracts (lanes 2 and 3). ctrl1 and ctrl2 are controls of age matched cataract tissues. GAPDH was used as protein loading control.

the data indicateed in Figure 3, CX46, which expressed in age-matched lens anterior epithelia and cataract fiber tissues (Figure 3A and 3B, lanes 2 and 3), was not detected in proband's lens tissues (Figure 3A and 3B; lane 1). In contrast, CX50 expressed in the lens fiber compartment of both proband and age-matched cataracts (Figure 3B). The alpha A-crystallins and alpha B-crystallins were detected in both anterior epithelia and fiber compartments of proband and age-matched cataracts (Figure 3A and 3B; lanes 1, 2 and 3). GAPDH was used for the protein loading control. Our results suggested that this novel frameshift mutation could impair CX46 protein expression.

DISCUSSION

In this paper, we found that a novel cytosine insertion mutation at site 1195 in Gja3cDNA, which caused a frameshift mutation in the C-terminal intracellular domain of the CX46 protein (cx46fs400), was the potential pathogenetic factor that was associated with this Chinese cataract family. This cx46fs400 mutation, which caused addition of 31 amino acids more to and amino acid substitution in CX46 intracellular C-terminal domain, may impair CX46 protein's structure and protein expression. Our results suggested that this novel cx46fs400 frameshift mutation should be closely related to the hereditary autosome dominant cataracts in this pedigree.

Genetic mutations in the CX46 gene are associated with various types of hereditary autosomal dominant cataracts. The 23 missense mutations, which are mostly located in the N-terminal or transmembrane domains, were reported to be associated with hereditary autosomal dominant cataracts[16]. Two frameshift mutations *e.g.* cx46fs380 and cx46fs397, which occured at the C-terminal tail of the CX46 protein, had been associated with autosomal dominant zonular pulverulent cataract[14,17]. Microinjection of cx46fs380 into *Xenopus oocytes* abrogated its membrane localization and disrupted its ability to form functional hemi-gap junction channels[18]. Knocking-in of cx46fs380 in mouse caused anterior nuclear cataracts with remarkable reduction in not only CX46 protein expression but also CX50 and alpha crystalline expression. Our data in Figure 1 revealed that a novel cytosine insert mutation in CX46 gene was observed in all the 5 tested affected individuals, but not in the 2 unaffected ones. Genetic-link analysis suggested that this novel frameshift cx46fs400 was associated with autosome heredity cataracts (Figure 1). Moreover, cx46fs400 protein was not detected in proband's anterior epithelia and fiber tissue compartments (Figure 3), suggesting this frameshift mutation in the C-terminus could be involved in the regulation of CX46 protein expression. However, unlike cx46fs380 mutation, cx46fs400 mutation had no regulatory effect on the expression of CX50 and alpha crystallins in the proband lens tissues (Figure 3). We were currently investigating whether knock-in of cx46fs400 affected the expression of CX50 and crystalline proteins in zebrafish model.

The carboxyl-terminal domain (223-435 aa) of CXs, which consists of the intracellular tail, plays an essential role in cellular signal transduction by interacting with other molecules. The C-terminal tail of CX43 alone is sufficient to induce cell motility[19]. S-nitrosylation of cysteine in C terminus of CX46 is involved in CX46 hemichannel function[20]. Moreover, CX46 or its family member CX50 is regulated by post-

translational phosphorylation. Wang and Schey[21] reported that there were 18 and 9 phosphorylation-regulated amino acids in bovine lens CX46 and CX50, respectively. Most of them were located at the C-terminal tail. Our sequence analysis results indicated that the amino acids normally phosphorylated in the wild type protein were completely changed in the cx46fs400 mutant (data not shown), which revealed the dis-regulation of phosphorylation in the cx46fs400 mutant. Previous studies have demonstrated that the C-terminal domain of lens CX46 and CX50 can interact with intercellular tight junction protein ZO-1[22-23]. Their interaction may be involved in gap junction turnover. Whether cx46fs400 mutant affect its association to OZ-1 is still under investigation in our lab.

Protein turnover of CXs is mainly regulated by two pathways, ubiquitin-proteasome and autophagy-lysosome pathways. It is reported that the CX43 C-terminal tail can interact with CIP75, an ubiquitin-associated (UBA) domain protein, facilitating CX43 degradation by the proteasome[24-25]. The CX50 frameshift mutant, which is associated with congenital recessive cataracts, was found to be degraded by the ERAD pathway and the proteasome[26]. Moreover, it was reported that the CX50 protein could be turned over when the cells were starved for 4h to induce autophagy. The CX50 protein was found to be co-localized with autophagic marker LC3, and its degradation could be inhibited by lysosomal inhibitor chloroquine. CX50/P88S mutant, which is associated with cataracts, was found to be degraded in the lysosome[27-28]. Our data in Figure 3 suggested that this novel cx46fs400 mutant was able to impair CX46 protein expression. Whether this novel mutant affects CX46 protein stability through ubiquitin-proteasome or lysosome pathways are still under studying in our lab.

We have identified a novel frameshift mutation (cx46fs400) in CX46 protein that is associated with hereditary autosomal dominant cataracts in a Chinese family. This mutation may inactivate CX46 channel function by impairing its protein expression.

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