·Basic Research·

A novel mutation of p.F32I in *GJA8* in human dominant congenital cataracts

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Abstract

• **AIM: To identify a causative mutation in a three-generation family with autosomal dominant congenital total cataract and dissect the molecular consequence of the identified mutation.**

• **METHODS: Clinical and ophthalmological examinations were performed on the affected and unaffected family members. Mutation were screened in recruited family members by polymerase chain reaction (PCR) of the two reported genes (*CRYAA* and *GJA8*) which were linked to human total cataracts and direct sequencing of the PCR product. The molecular consequences of the identified mutation was dissected. The plasmids carrying wild-type and mutant mouse *ORF* of *Gja8*, coding for connexin 50 (Cx50), were generated and ectopic expressed in 293 cells. Recombinant protein expression and cellular localization of recombinated Cx50 were assessed by confocal microscopy.**

• **RESULTS: Clinical and ophthalmological examinations were performed on the affected and unaffected family members. Mutation were screened in recruited family members by PCR of the two reported genes (*CRYAA* and *GJA8*) which were linked to human total cataracts and direct sequencing of the PCR product. The molecular consequences of the identified mutation was dissected. The plasmids carrying wild-type and mutant mouse *ORF* of *Gja8*, coding for Cx50, were generated and ectopic expressed in 293 cells. Recombinant protein expression and cellular localization of recombinated Cx50 were assessed by confocal microscopy.**

• **CONCLUSION: This study has identified a novel cataract mutation in *GJA8*, which adds a novel mutation to the existing spectrum of Cx50 mutations with cataract. The molecular consequences of p.F32I mutation in *GJA8* exclude instability and the mislocalization of mutant Cx50 protein.**

• **KEYWORDS:** cataracts; mutation; connexin 50; *GJA8*; molecular consequences
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INTRODUCTION

Cataracts are generally defined as an opacity or loss of optical uniformity of the crystalline lens [1]. Types of cataracts can be defined by the age at onset: congenital, juvenile, presenile and senile or age-related cataracts [2]. Congenital cataracts are responsible for 10% -30% of childhood blindness [3]. Around one fifth of congenital cataracts have a family history. Hereditary cataracts are a clinically and genetically heterogeneous lens disease and they causes a significant proportion of visual impairment and blindness in childhood [4]. Clinically, they can be subdivided according to their anatomical location within the lens, their appearance, and most commonly by a combination of these two parameters [5]. Genetically, there are more than 41 independent loci to which isolated or primary cataracts have been mapped and 30 distinct genes responsible for nonsyndromic hereditary cataracts have been identified (Table 1)[4,6].

In the present study, we sought to identify the genetic defect of autosomal dominant congenital total cataracts in a Chinese family. Mutations in two selected genes, *CRYAA* and *GJA8*, were directly screened in recruited family

Table 1 Disease genes and mapped locus for cataracts

Phenotype	Location	Locus/gene	OMIN ID
Cataract1 multiple types	1q21.2	CTRCT1, GJA8	600897
Cataract2 multiple types	2q33.3	CTRCT2, CRYGC	123680
Cataract3 multiple types	22q11.23	CTRCT3, CRYBB2	123620
Cataract4 multiple types	2q33.3	CTRCT4, CRYGD	123690
Cataract5 multiple types	16q22.1	CTRCT5, HSF4	602438
Cataract6 multiple types	1p36.13	CTRCT6, EPHA2	176946
Cataract7	17q24	CTRCT7	115660
Cataract8 multiple types	1pter-p36.13	CTRCT8	115665
Cataract9 multiple types	21q22.3	CTRCT9, CRYAA	123580
Cataract10 multiple types	17q11.2	CTRCT10, CRYBA1	123610
Cataract11 multiple types	10q24.32	CTRCT11, PITX3	602669
Cataract11 syndromic	10q24.32	CTRCT11, PITX3	602669
Cataract12 multiple types	3q22.1	CTRCT12, BFSP2	603212
Cataract13 with adult phenotype	6p24.3-p24.2	CTRCT13, GCNT2 Ii	600429
Blood group Ii	6p24.3-p24.2	CTRCT13, GCNT2 Ii	600429
Adult phenotype without cataract	6p24.3-p24.2	CTRCT13, GCNT2 Ii	600429
Cataract14 multiple types	13q12.11	CTRCT14, GJA3	605728
Cataract15 multiple types	12q13.3	CTRCT15, MIP	154050
Cataract16 multiple types	11q23.1	CTRCT16, CRYAB	123590
Cataract17 multiple types	22q12.1	CTRCT17, CRYBB1	600929
Cataract18 autosomal recessive	3p21.31	CTRCT18, FYCO1	607182
Cataract19	19q13.41	CTRCT19, LIM2	154045
Cataract20 multiple types	3q27.3	CTRCT20, CRYGS	123730
Cataract22 autosomal recessive	22q11.23	CTRCT22, CRYBB3	123630
Cataract23	22q12.1	CTRCT23, CRYBA4	123631
Cataract24 anterior polar	17p13	CTRCT24, CTAA2	601202
Cataract25	15q21-q22	CTRCT25	121015
Cataract26 multiple types	9q13-q22	CTRCT26	605749
Cataract27 nuclear progressive	2p12	CTRCT27	607304
Cataract28 age-related cortical	6p12-q12	CTRCT28, ARCC1	609026
Cataract29 coralliform	2pter-p24	CTRCT29	115800
Cataract30 pulverulent	10p13	CTRCT30, VIM	193060
Cataract31 multiple types	20q11.22	CTRCT31, CHMP4B	610897
Cataract33	20p12.1	CTRCT33, BFSP1	603307
Cataract34 multiple types	1p34-p32.2	CTRCT34	612968
Cataract35 congenital nuclear	19q13	CTRCT35	609376
Cataract36	9q22.33	CTRCT36, TDRD7	611258
Cataract37 autosomal dominant	12q24.2-q24.3	CTRCT37	614422
Cataract38 autosomal recessive	7q34	CTRCT38, AGK	610345
Cataract39 multiple types autosomal dominant	2q34	CTRCT39, CRYGB	123670
Cataract40 X-linked	Xp22.13	CTRCT40, NHS	300457
Cataract41	4p16.1	CTRCT41, WFS1	606201
Cataract42	2q35	CTRCT42, CRYBA2	600836
Cataract pulverulent or cerulean with or without microcornea	16q23.2	MAF, CCA4	177075

members, because mutations in both genes have been identified in our previous studies and, furthermore, both are reported to be linked with cases of total cataracts [7-8]. In this family, a mutation in the *GJA8* gene, which encodes connexin 50 (Cx50), was identified. To dissect the molecular consequence of the identified mutation, the wild-type (WT) and mutant mouse *Gja8* coding sequences were inserted into eukaryotic gene expression vectors, respectively. Ectopic expression and cellular localization of the WT and mutant mouse *Gja8* proteins were assessed by confocal microscopy.

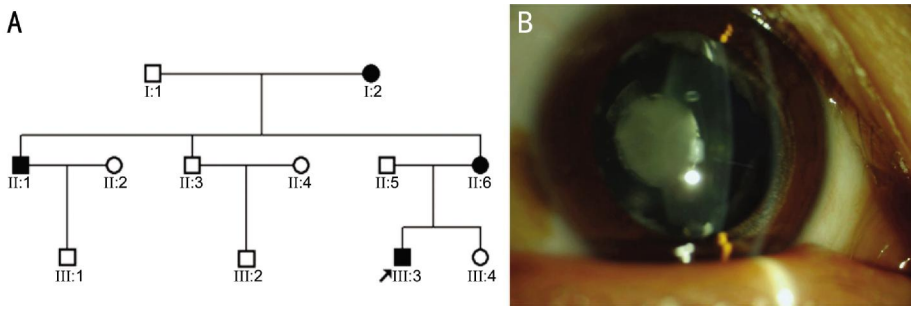

Figure 1 Cataract pedigree and phenotype A: Cataract pedigree. Squares and circles represent males and females, respectively. White and black lines denote unaffected and affected status, respectively. Arrow indicates the proband recruited in the family; B: Photographs of affected individuals of this family. The phenotype of the proband (III: 3) is diagnosis as total cataract because of bilateral complete opacification of the fetal nucleus and the cortex.

Table 2 Primers used for mutation screening of *CRYAA* and *GJA8*

Gene	Exon	Direction	Sequence	Product size (bp)	Temperature(℃)
CRYAA	1	Forward	CTCGGGGACAGTCCGTGCAG	670	60
	1	Reverse	TGATGGAGGAAAGCAAAGG		
	2	Forward	CGGCCCACCAGTAGCATT	616	58
	2	Reverse	GATCCCTGAGCCACATTAGC		
	3a	Forward	GATCCCTGAGCCACATTAGC	560	60
	3a	Reverse	GTGAGAAGGTGGAAAGGAAA		
	3b	Forward	CGGGAGGAGAAGCCCACC	702	60
	3b	Reverse	CGGGAGGAGAAGCCCACC		
GJA8	1	Forward	GCCAATCCAGTCATATCTGC	400	58
	1	Reverse	CCATCCTGCTGATTATTCCA		
	2a	Forward	GCAACTTGGAAAGGAGAGGT	658	58
	2a	Reverse	GCGGTACAGAGGCAGGAT		
	2b	Forward	GGCAGCAAAGGCACTAAG	571	60
	2b	Reverse	CGTAGGAAGGCAGTGTCTCT		
	2c	Forward	TGTCTCCTCCATCCAGAAAG	611	58
	2c	Reverse	GATCATGTTGGCACCTTTTC		
Gja8	F32I	Forward	ctcacagtgctcttcatcatccgcatcctcatc		58
	F32I	Reverse	gatgaggatgcggatgatgaagagcactgtgag		

SUBJECTS AND METHODS

Patients This study approved by the Ethics Committee of the Eye Hospital, Wenzhou Medical University. It conformed to the tenets of the Declaration of Helsinki. We obtained written informed consent from all participating individuals (or their guardians). Clinical examinations were performed by ophthalmologists. The recruited family comprised 4 affected individuals from a three generation pedigree (Figure 1A). A 5 mL venous blood sample was drawn from the recruited family members. Genomic DNA was extracted by standard techniques.

Mutation Screening and Analysis We designed a set of eight primer pairs to amplify all the exons and intron/exon boundaries of the two candidate genes (*CRYAA* and *GJA8*). Oligonucleotide primer pairs and polymerase chain reaction (PCR) information for amplification are described in Table 2. PCR products were sequenced with Sanger sequencing. We obtained Cx50 sequence from various species and two Cx family members, Cx40 and Cx43 from *Homo sapiens*. Multiple-sequence alignment of Cx50 in these species was generated with DNAMAN biosoftware. Furthermore, we used different online bioinformatics software, PolyPhen-2 (PolymorphismPhenotyping-v2) program (http://genetics.bwh.harvard.edu/pph2), Panther (http://www.pantherdb.org/tools/csnpScoreForm.jsp), Sorting Intolerant from Tolerant (SIFT) and Pmut (http:// mmb2.pcb.ub.es:8080/PMut/) to predict whether the amino acid substitution in Cx50 could have a phenotypic effect.

Cloning and Expression of Proteins Recombinant mouse WT and mutant Cx50 coding fragments were prepared as previously described [8]. Briefly, we obtained the coding sequence for WT and mutant Cx50 by PCR (Table 2). Then the coding consequences were inserted into vector pEGFPc1 to generate pEGFPc1-WT and pEGFPc1-MUT with *Bgl II* and *Xba I*, respectively. Desired specific sequence in the constructs were confirmed by DNA sequencing.

Table 3 Domains of Cx46 and Cx50 and the corresponding mutations

Location	Cx46			Cx50		
	Position	Length	Mutations	Position	Length	Mutations
N	1-19	19	4	1-23	23	1
TM1	20-40	21	3	24-46	23	7
E1	41-76	36	11	47-76	30	10
TM2	77-97	21	1	77-99	23	5
CL	98-152	55	1	100-150	51	0
TM3	153-173	21	0	151-173	23	0
E2	174-201	28	5	174-204	31	7
TM4	202-222	21	1	205-227	23	0
C	223-435	213	3	228-433	206	7

The recombinant plasmids were expressed and the expression products were investigated in human embryonic kidney (HEK-293, http://www.atcc.org/products/all/CRL-1573.aspx) cells. Briefly, we seeded cells at day 0 and performed transfection at day 1. Cells were fixed with 4% paraformaldehyde at day 3 for transient gene expression. To label plasma membrane, the cells were transfected with plasmid harboring membrane-tagged expressions of RFP as a control. To label the cellular nuclei, DAPI was used. The treated cells were observed under laser confocal microscope.

RESULTS

Clinical Data The proband is a 12-year-old male (III: 3, Figure 1A). His phenotype is bilateral complete opacification of the fetal, infantile, and adult nucleus and the cortex, and its phenotype is of total cataract (Figure 1B), with low vision (20/100). The proband suffered from a bilateral symmetric opacity of the lens since birth, but did not progress with age. There was no family history of other ocular or systemic abnormalities. Other family members have similar cataracts patterns.

Mutation in *GJA8* Identification and Analysis Directional sequencing of the coding regions of the candidate genes showed only one heterozygous change in exon 2 (T>C) of *GJA8*, at position c.94 of the Cx50 gene in the proband and his mother (Table 2, Figure 2A). This mutation leads to the replacement of a highly conserved phenylalanine with isoleucine at the 32 amino acid position (p.F32I). The remainder of the coding sequence did not show any sequence changes.

Multiple-sequence alignments of Cx50 in various species were obtained. We found that codon 32 is located within a highly conserved region (Figure 2B). Furthermore, we used online bioinformatics software to predict and all of these results strongly suggest that p.F32I variation is a pathogenic mutation (data not shown).

We searched this mutation in multiple databases, including the 1000 Genome, HapMap, dbSNPs (v130) and 1402 in-house exome data. We didn't find it was present in one of these databases. Taken together, all these data demonstrated that the p.F32I substitution is a causative mutation.

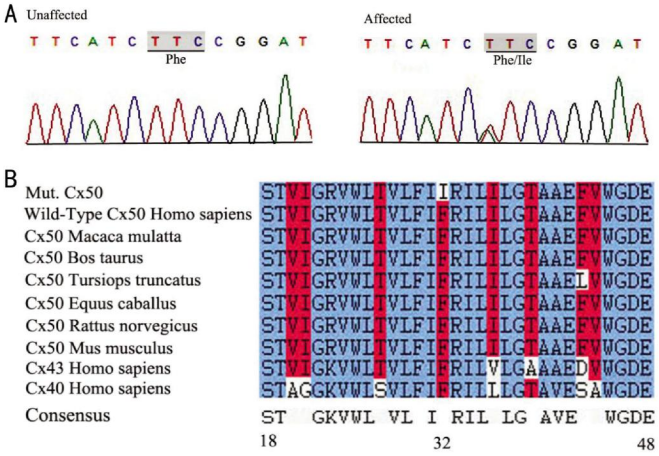

Figure 2 Mutation analysis A: DNA sequence chromatograms analysis. DNA sequence chromatograms of unaffected members and affected members in an autosomal dominant total cataract family. A single base alteration of C>A transversion in exon 1 causes a conservative substitution of Phe to Ile at codon 32 (p.F32I); B: Multiple protein sequence alignments. Multiple-sequence alignment revealed p.F32I located within a highly conserved region. The "mut." sequence indicates the sequence identified in the present study.

Mutation Effects on the Protein To dissect the molecular consequence of the mutation identified in this family, we generated the patients-associated GFP-tagged Cx50 mutants. Specifically, genes coding for WT and mutant Cx50 were inserted into eukaryotic gene expression vectors (pEGFPc1), respectively.

Confocal immunocytochemical analysis showed that both WT and mutant Cx50 could be ectopically expressed (Figure 3), which indicated the mutation may not affect protein stability. Furthermore, both the WT and mutant Cx50 were distributed in the cytoplasm and plasma membrane. No significant difference between the localization of WT and mutant connexin (Cx) was observed, which reveals that the molecular consequences of p.F32I mutation in *GJA8* excludes the mislocalization of mutant Cx50 protein.

DISCUSSION

Diverse cataract mutations have been identified in humans[7-9]. Proteins in the lens can be divided into soluble and insoluble

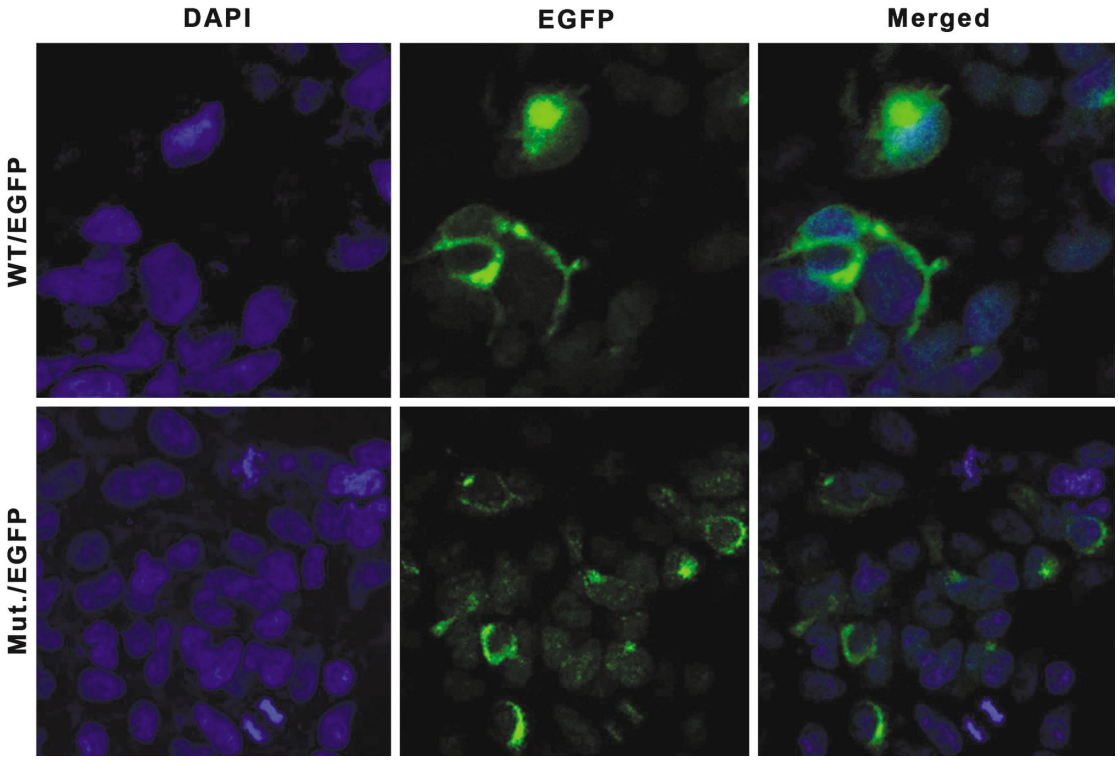

Figure 3 Ectopic expression and location of p.F32I mutant Cx50 Plasmids coding for Cx50-WT, mutant (p.F32I) were transfected into 293 cells and corresponding images were obtained under the microscope. No significant difference of the ectopic gene expression and location between the WT and mutant Cx50.

proteins. The majority (90%) of the soluble proteins in the lens are crystallins. Insoluble proteins include membrane proteins, beaded filament proteins, and protein aggregation of crystals [10]. Gap junctional intercellular communication (GJIC) plays an important role in the maintenance of tissue independence and homeostasis in multicellular organisms[11]. The avascular lens obtains nutrients, maintains metabolic balance, and facilitates ion exchange through gap junction [12]. Therefore, it is no surprise that mutations in the genes coding for the Cx molecules comprising gap junctions will lead to cataracts. There are three Cxs (Cx43, Cx46 and Cx50) identified in the human lens [12]. Cx46 and Cx50 have been found to be the most abundant Cxs in lens fiber cells, which has been reported to be causative genes in the cases of cataracts. Because mutations in *GJA1*, coding for Cx43, cause human oculodentodigital dysplasia (ODDD)[13], it is a distinct disease from cataracts, we excluded it as the candidate gene in this study. In our recent study, we identified a novel mutation in Cx50 associated with a phenotype of total cataract[7]. Therefore, we considered Cx50 a reasonable candidate gene to scan for mutations.

All of the Cx proteins contain four transmembrane domains (TM1-TM4), two extracellular loops (E1 and E2), a cytoplasmic loop (CL) between TM2 and TM3, and cytoplasmic N-terminal (N) and C-terminal (C) domains, and amino acid composition of each region is very similar (Table 3). To date, there are 37 mutations identified in human Cx50 and Cx46, respectively (Tables 1, 4). It is an open question

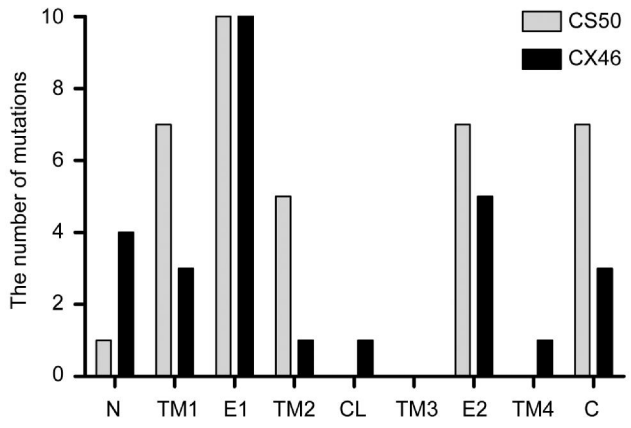

Figure 4 Reported mutations in human *GJA3* and *GJA8* All reported human *GJA3* and *GJA8* mutations associated with cataracts have been mapped in the domain of Cx46 and Cx50 protein, respectively. Cx proteins contain four transmembrane domains (TM1-TM4), two extracellular loops (E1 and E2), a CL between TM2 and TM3, and cytoplasmic N-terminal (N) and C-terminal (C) domains.

whether there is any mutation-rich region. To address this question, we mapped reported mutations to these domains and found the two extracellular loops of Cx represent a mutation-rich region (47.8%) for the reported mutations (Figure 4). This analysis also revealed that the extracellular loops are crucial for the generation of the gap junction, highlighting that more attention should be paid to the exons coding for extracellular loops when screening for mutations causing cataracts.

Table 4 Summary of identified mutations in *GJA8*

Location	DNA change	Coding change	Inheritance	Origin	Cataract phenotype
N	c.68G>C	p.R23T	AD	Iran	Nuclear
TM1	c.92T>C	p.I31T	AD	China	Nuclear
TM1	c.94T>A	p.F32I	AD	China	Total
TM1	c.116C>G	p.T39R	Sporadic	China	Total
TM1	c.131T>A	p.V44E	AD	India	Total
TM1	c.134G>C	p.W45S	AD	India	"Jellyfish-like"
TM1	c.137G>T	p.G46V	AD	Palestine	Total
TM1	c.136G>A	p.G46R	AD	China	Total
E1	c.139G>C	p.D47H	AD	China	Zonular nuclear pulverulent
E1	c.139G>T	p.D47Y	AD	China	Nuclear
E1	c.139G>A	p.D47N	AD	UK	Nuclear pulverulent
E1	c.139G>A	p.D47N	AD	China	Nuclear
E1	c.139G>A	p.D47N	AD	China	Nuclear
E1	c.142G>A	p.E48K	AD	Pakistan	Zonular nuclear pulverulent
E1	c.191T>G	p.V64G	AD	China	Nuclear
E1	c.200A>G	p.D67G	AD	USA	No answer
E1	c.218C>T	p.S73F	AD	Denmark	Nuclear
E1	c.226A>G	p.R76C	AD	USA	No answer
TM2	c.235G>C	p.V79L	AD	India	"Full moon" with Y-sutural opacities
TM2	c.262C>T	p.P88S	AD	UK	Zonular pulverulent
TM2	c.262C>A	p.P88Q	AD	UK	Lamellar pulverulent
TM2	c.262C>A	p.P88Q	AD	India	Balloon-like, sutural
TM2	c.264C>A	p.P88T	AD	China	Total
E2	c.566C>T	p.P189L	AD	Denmark	Nuclear
E2	c.649G>A	p.V196M	AR	India	No answer
E2	c.592C>T	p.R198W	AD	China	No answer
E2	c.593G>A	p.R198Q	AD	India	Posterior subcapsular
E2	c.658C>T	p.P199S	AD	India	No answer
E2	c.601G>A	p.E201K	AD	China	Lamellar
E2	c.608insA	p.T203NfsX47	AR	India	Total
C	c.741T>G	p.I247M	AD	Russia	Zonular pulverulent
C	c.741T>G	p.I247M	Sporadic	Germany	No answer
C	c.767insG	p.A256GfsX124	AR	Germany	Nuclear
C	c.773C>T	p.S258F	AD	China	Nuclear
C	c.836C>A	p.S259Y	AD	Denmark	No answer
C	c.823G>A	p.V275I	Complex	China	Age-related cortical
C	c.827C>T	p.S276F	AD	China	Nuclear pulverulent
C	c.842T>C	p.L281C	AD	India	Lamellar/zonular

Several pathogenic mutations in Cx50 and Cx46 have been identified within the TM1 in human families with congenital cataracts, including p.I31T substitution of Cx50 and p.F32L, p.R33L of Cx46, and the novel missense mutation in Cx50 (p.F32I) reported in this work. The amino acid residues at position 31, 32, 33 of Cx are highly conserved across various species. We speculate that these sites may play an important role in maintaining eye lens transparency, because mutations at these sites result in opacification of the lens. It is consistent with the conclusion that the domain with these specific residues may participate in the oligomerization into connexon hemichannels and is also essential for the correct transport of the protein into the plasma membrane[14-15].

Theoretically, different mutations in different domains of Cx50 may have the same specific consequence. We observed distinctive recombinant protein expression pattern of p.P88T mutant Cx50 protein, compared with the WT [7]. On the contrary, there is no significant difference between the WT and p.F32I mutant Cx50 protein. Specifically, no protein instability or mislocalization of ectopic mutant Cx50 protein has been observed. Here we provide direct evidence to support those different mutations in different domain of Cx50 may have a specific consequence.

So far, it is a challenge for the molecular diagnosis of cataracts, the reason for it is that the relatively large numbers (30) of disease genes. It makes cataracts as one of the most complicated monogenic disorders. By taking advantage of phenotype and genotype relationship, here we showed that using a functional cloning approach, we could successfully identify the causative gene for hereditary cataracts. From the human development process, it would not be surprising that mutation in *GJA8* will cause human total cataract, because Cx molecules composing GJIC play an extremely important role in the maintenance of tissue independence and homeostasis in multicellular organisms, thus patients with *GJA8* mutations have a total cataract phenotype. This case will be valuable for cataract genetic counseling of the family members and prenatal diagnosis. It also highlights that it may be meaningful to directly screen these Cx coding genes for mutations in families with human hereditary total cataracts, since only eight genes (*CRYAA*, *CRYBB2*, *GJA8*, *GJA3*, *EPHA2*, *PITX3*, *MIP* and *HSF4*) have been linked with this total cataracts[7-8,10,16-24].

In summary, by taking advantage of phenotype and genotype relationship, we report a novel missense mutation in Cx50 in a Chinese family with autosomal dominant congenital total cataract. It also highlights that TM1 is an important structural domain in *GJA8* relative to cataract formation. We also exclude instability and the mislocalization of mutant Cx50 protein as the corresponding molecular consequences. Further study is needed to provide insights into the molecular pathogenic mechanisms caused by new cataract-causing mutations in Cx50.

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