

A Missense Mutation in *GJA8* Encoding Connexin 50 in a Chinese Pedigree with Autosomal Dominant Congenital Cataract

Lusi Zhang,^{1,2} Youling Liang,^{1,2} Yedi Zhou,^{1,2} Huilan Zeng,^{1,2} Songbai Jia^{1,2} and Jingming Shi^{1,2}

¹Department of Ophthalmology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

²Hunan Clinical Research Center of Ophthalmic Disease, Changsha, Hunan, China

Congenital cataract is leading cause of visual impairment and blindness in children worldwide. Approximately one-third of congenital cataract cases are familial, whose genetic etiology can be distinguished by targeted exome sequencing. Here, a three-generation congenital cataract pedigree was recruited, and physical and ophthalmologic examinations were taken. Targeted exome sequencing of 139 cataract-related genes was performed on the proband III:1. Sanger sequencing was used to validate the presence of variation identified via exome sequencing in family members and 200 controls. Conservative and functional prediction was performed with bioinformatic tools. We, thus, found a heterozygous missense mutation c.10T>A (p.W4R) in *gap junction protein alpha 8 (GJA8)* in the patients. However, this mutation was not present in normal family members and 200 unrelated controls. The *GJA8* gene encodes a gap junction protein, connexin 50 (Cx50), in lens fibers that provide channels for exchange of ions and small molecules between adjacent cells. Conservative and functional prediction suggests that the W-to-R substitution at codon 4 may impair the function of the human Cx50 protein. Accordingly, we analyzed the distribution of Flag-tagged mutant Cx50 protein in HeLa cervical cancer cells. Immunofluorescent staining showed that the W-to-R substitution impaired Cx50 trafficking to the plasma membrane to form the gap junction. In conclusion, c.10T>A (p.W4R) in *GJA8* is the newly identified genetic cause of familial congenital cataract. The W-to-R substitution near the amino-terminus may alter the localization of mutant Cx50, thereby impairing gap junction formation, which is the molecular pathogenic mechanism of this mutation.

Keywords: congenital cataract; connexin 50; gap junction; *GJA8*; targeted exome sequencing

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Introduction

The lens in the eye help to transmit and focus light onto the retina, which is received by photoreceptors and converted into visual signals. Opacity or cloudiness of the crystalline lens would lead to cataract. Congenital cataract is a heterogeneous lens disorder that usually presents at birth or during early childhood (Churchill and Graw 2011; Li et al. 2016a). The prevalence of congenital cataract is one to six per 10,000 live births, which is the primary cause of treatable childhood blindness worldwide (Pi et al. 2012).

Congenital cataract may occur as an isolated defect or be combined with other anterior segment developmental disorders, such as microphthalmia and aniridia (Bremond-Gignac et al. 2010; Zhou et al. 2010; Gillespie et al. 2015).

Furthermore, the disease may also serve as part of multi-system genetic disorders, such as chromosome abnormalities, Lowe syndrome, and Nance-Horan Syndrome (Coccia et al. 2009; Daskalakis et al. 2010; Forbes et al. 2016). These results suggest markedly heterogeneous phenotypes of the disease. Congenital cataract might be inherited or caused by certain intrauterine insults. About one-third of congenital cataract cases are familial, the majority of which are autosomal dominant, and some are autosomal recessive or X-linked (Haargaard et al. 2005). Thus, genetic diagnosis can be successfully provided to patients and families for clinical counseling and prenatal diagnosis.

To date, over 40 genes have been reported to cause congenital cataract. They are often translated to a variety of lens structural proteins, such as crystallin, gap junction,

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Correspondence: Songbai Jia, M.D., Department of Ophthalmology, The Second Xiangya Hospital, Central South University, 139 Renmin Middle Road, Changsha, Hunan 410011, China.

e-mail: jsb88cn@csu.edu.cn

Jingming Shi, M.D., Department of Ophthalmology, The Second Xiangya Hospital, Central South University, 139 Renmin Middle Road, Changsha 410011, China.

e-mail: sjm93cn@csu.edu.cn

lens membrane proteins, intermediate filament, and ocular developmental related transcription factors (Ma et al. 2016). Among these genes, crystallin genes *CRYAA* (crystallin alpha A), *CRYAB* (crystallin alpha B), *CRYBA1* (crystallin beta A1), *CRYBB1* (crystallin beta B1), *CRYBB2* (crystallin beta B2), *CRYBB3* (crystallin beta B3), *CRYBA2* (crystallin beta A2), *CRYBA4* (crystallin beta A4), *CRYGC* (crystallin gamma C), *CRYGD* (crystallin gamma D), and *CRYGS* (crystallin gamma S) contribute to approximately half of the pathogenic mutations, whereas a quarter of the mutations were derived from connexin genes *GJA3* (gap junction protein alpha 3) and *GJA8* (gap junction protein alpha 8) (Ren et al. 2017). Moreover, there are many genes that lead to syndromic congenital cataract with associated systemic features. Owing to high heterogeneity of the genetic basis and phenotypes, traditional Sanger sequencing platform is no longer an ideal method to detect congenital cataract related mutations. Targeted next-generation sequencing (NGS) would be a powerful tool to detect pathogenic variants in congenital cataract patients.

In our study, we applied targeted exome sequencing-based mutant detection to our congenital cataract pedigree, and found out a heterozygous missense mutation (c.10T>A, p.W4R) of *GJA8* in our patients, which is co-segregated with cataract phenotype in the family and was not retrieved in 200 normal controls as well as dbSNP database. Moreover, conservative and functional prediction revealed that p.W4R mutation probably impacts the normal function of human GJA8 protein (also named as connexin 50, Cx50). Finally, immunofluorescent staining of Flag-tagged Cx50 protein (wildtype, WT) and mutant protein suggested that the W-to-R substitution at codon 4 affected gap junction formation in HeLa cells, which may be responsible for the onset of cataract.

Materials and Methods

Subjects

An autosomal dominant cataract pedigree was recruited from the Second Xiangya Hospital, Hunan, China, which consisted of six members (two males and four females) from three generations (Fig. 1A). Normal controls (n = 200) were recruited mainly from the hospital and other volunteers. Peripheral blood (2-3 ml) was obtained from our family members as well as normal controls. The standard phenol-chloroform method was performed to extract genomic DNA from peripheral blood. All of the pedigree members and controls gave their written informed consent complying with the Declaration of Helsinki principles. This study was approved by the Second Xiangya Hospital Ethics Committee.

Targeted exome sequencing and mutation prediction

DNA of proband III:1 was used for targeted exome sequencing of 139 cataract-related genes. Library construction was performed by the customized Roche NimbleGen SeqCap EZ System (Roche, Madison, Wisconsin, USA), and 90-cycles Paired-end sequencing was conducted on Illumina HiSeq2500 Analyzers (Illumina, San Diego, California, USA). Read mapping and variant analysis procedures were as described previously (Li et al. 2016b). On the basis of

dbSNP database (www.ncbi.nlm.nih.gov/SNP/), Exome Variant Server database (<http://evs.gs.washington.edu/EVS/>), the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>) and 1,000 Genomes (<http://browser.1000genomes.org/index.html>) annotation, the high-frequency (minor allele frequency ≥ 0.01) polymorphism variations were excluded. To perform functional predictions, we used the software Scale-invariant feature transform (SIFT), Polymorphism Phenotyping v2 (PolyPhen 2), and MutationTaster to score these variants. For conservative prediction Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was made.

Sanger sequencing of implicated gene

PCR amplification and Sanger sequencing were used to validate the variation in *GJA8* identified via exome sequencing. Primer sequences are shown as below: Forward primer 5'-tgcttcagaccattgt gaag-3', Reverse primer 5'-CTCGTCGTAGCAGACGTTCTC-3'. PCR was performed in a 10 μ l reaction mixture containing 5 μ l 2 \times TaKaRa Taq™ HS Perfect Mix (TaKaRa, Dalian, Liaoning, China), 30 ng each primer, and 30 ng of genomic DNA. The amplification conditions consisted of an initial step of denaturation at 94°C for 30 sec, followed by 33 cycles of denaturation at 94°C for 5 sec, annealing at 60°C for 20 sec and extension at 72°C for 20 sec. A final extension was performed at 72°C for 7 min.

Connexin 50 constructs and HeLa cell transfections

Recombinant human Cx50-coding sequence inserted in pEGFP-C1 vector was a gift from Prof. Yihua Zhu (Fujian Medical University, Fuzhou, China). Cx50 coding sequence was cloned into pcDNA 3.1/myc-His (-) B vector (Invitrogen, Carlsbad, CA, USA). And Flag-tag (N-DYKDDDDK-C) was added into C-terminal of Cx50-coding sequence. QuikChange Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA, USA) was used to construct the mutant Cx50 W4R plasmid. The Cx50 Wildtype (WT) and W4R plasmids were sequenced to verify the inserted sequence and the target mutation.

HeLa cells were cultured in high-glucose Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco, Carlsbad, CA, USA) with 10% Fetal Bovine Serum (FBS) (Gibco, Carlsbad, CA, USA). Cx50 WT-Flag or Cx50 W4R-Flag was transfected into HeLa cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions.

Immunofluorescent staining

Celular distribution and gap junction formation of Cx50 protein were observed on Flag-tagged WT and W4R in HeLa cells using a confocal laser scanning microscope (Leica, Wetzlar, Germany). After 36-48 hrs of transfection, cells were fixed with 3.7% paraformaldehyde/10% sucrose for 15 min, and permeabilized with 0.1% Triton X-100 in 1 \times Phosphate Buffered Saline (PBS) for 10 min. Then cells were blocked with 5% Bovine Serum Albumin (BSA) in 1 \times PBS for 30 min, and incubated with polyclonal rabbit Flag antibody (Sigma-Aldrich F7425, St. Louis, MO, USA) overnight. Rinse the cells for a couple times with PBS, and block again. Secondary antibody Cy3 labeled anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was incubated for 1 hour. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was used to staining cellular nucleus.

Table 1. Summary of the targeted exome sequencing results.

Sample	III-1
Targeted Genes	139 genes: <i>GJA8</i> , <i>CRYGC</i> , <i>CRYBB2</i> , <i>CRYGD</i> , <i>HSF4</i> , <i>EPHA2</i> , <i>CRYAA</i> , <i>CRYBA1</i> , <i>PITX3</i> , <i>BFSP2</i> , <i>GCNT2</i> , <i>GJA3</i> , <i>MIP</i> , <i>CRYAB</i> , <i>CRYBB1</i> , <i>FYCO1</i> , <i>LIM2</i> , <i>CRYGS</i> , <i>MAF</i> , <i>CRYBB3</i> , <i>CRYBA4</i> , <i>VIM</i> , <i>CHMP4B</i> , <i>BFSP1</i> , <i>TDRD7</i> , <i>AGK</i> , <i>CRYGB</i> , <i>WFS1</i> , <i>CRYBA2</i> , <i>CLPB</i> , <i>JAG1</i> , <i>ALMS1</i> , <i>BBIP1</i> , <i>BBS2</i> , <i>MED25</i> , <i>NOD2</i> , <i>TGFBI</i> , <i>ABHD5</i> , <i>GJB6</i> , <i>CC2D2A</i> , <i>ERCC8</i> , <i>ERCC6</i> , <i>LONP1</i> , <i>MYH9</i> , <i>NR2E3</i> , <i>SRD5A3</i> , <i>COL18A1</i> , <i>KCNJ13</i> , <i>OCRL</i> , <i>RAB3GAP2</i> , <i>NDP</i> , <i>PQBP1</i> , <i>CREBBP</i> , <i>HSPG2</i> , <i>PIK3R1</i> , <i>DHCR7</i> , <i>VCAN</i> , <i>EPG5</i> , <i>RAB3GAP1</i> , <i>RAB18</i> , <i>TBC1D20</i> , <i>FBN1</i> , <i>EBP</i> , <i>DKC1</i> , <i>IARS2</i> , <i>GALK1</i> , <i>GALT</i> , <i>CAV1</i> , <i>OPA3</i> , <i>COL4A3</i> , <i>COL4A4</i> , <i>ALDH18A1</i> , <i>JAM3</i> , <i>PLOD3</i> , <i>ADAMTSL4</i> , <i>WDR19</i> , <i>ALX3</i> , <i>ABHD12</i> , <i>TBC1D24</i> , <i>P3H2</i> , <i>FTL</i> , <i>PEX1</i> , <i>PEX5</i> , <i>PEX2</i> , <i>PEX7</i> , <i>LOXLI</i> , <i>PTH</i> , <i>GCM2</i> , <i>LRP5</i> , <i>LMX1B</i> , <i>MVK</i> , <i>GNAS</i> , <i>POLG</i> , <i>SLC4A4</i> , <i>PHGDH</i> , <i>CYP27A1</i> , <i>OAT</i> , <i>SLC2A1</i> , <i>SLC16A12</i> , <i>CNGB3</i> , <i>IKBKKG</i> , <i>NF2</i> , <i>CLN3</i> , <i>COL2A1</i> , <i>COL11A1</i> , <i>BEST1</i> , <i>IMPG2</i> , <i>RBP3</i> , <i>ITM2B</i> , <i>GUCY2D</i> , <i>FAM126A</i> , <i>ETFA</i> , <i>ETFB</i> , <i>ETFDH</i> , <i>CTDPI</i> , <i>SLC33A1</i> , <i>B3GALNT2</i> , <i>POMK</i> , <i>POMT1</i> , <i>POMT2</i> , <i>POMGNT1</i> , <i>FKTN</i> , <i>FKRP</i> , <i>ISPD</i> , <i>ALG2</i> , <i>DPAGT1</i> , <i>GFER</i> , <i>GJA1</i> , <i>SILI</i> , <i>ZNF335</i> , <i>ERCC3</i> , <i>ERCC2</i> , <i>ERCC5</i> , <i>DAG1</i> , <i>TRAPPC11</i> , <i>GNPAT</i> , <i>PAX6</i> , <i>BCOR</i> , <i>OTX2</i>
Targeted region (bp)	351,984
Targeted region mapped (%)	99.78
Mean depth (×)	119.03
Mean depth > 30× (%)	97.31

Database as well, which provide evidence that the mutation we detected was not a SNP, nor reported by other groups. Thus, our results suggest that *GJA8* c.10T>A is not a rare polymorphism in the population, but a pathogenic mutation for our autosomal dominant congenital cataract family.

Conservative and functional prediction on c.10T>A (p.W4R) missense variant in *GJA8*

To investigate the possible influence of the missense mutation on *GJA8* gene product Cx50 protein, we performed the conservative and functional prediction through Clustal Omega, MutatinTaster, Polyphen-2 and SIFT on our mutation. Clustal Omega result demonstrated asterisks in amino acid codon 4 (Fig. 1C), showing the identical amino acid on this site between different species and high conservation on codon 4. As showed in Table 2, MutationTaster predicted c.10T>A as a disease causing nucleotide substitution. Polyphen-2 score showed a pathogenic mutation on

p.W4R, which predicted possible impact of the amino acid substitution on the structure and function of Cx50. SIFT results predicted that the W-to-R substitution at codon 4 will damage protein function, suggesting that the c.10T>A (p.W4R) mutation probably impairs the function of the mutant Cx50 protein.

Different gap junction formation pattern of Cx50 W4R mutation

Gap junction channels consist of connexin protein subunits. Cx50 was reported to modulate diffusion of molecules between lens fiber cells and regulate electrical properties (Xin et al. 2010) on the plasma membrane. To further investigate the pathophysiological function of our pathogenic mutation, we transiently expressed Cx50 WT-Flag and Cx50 W4R-Flag in HeLa cells. Immunofluorescent staining showed that Cx50 WT were localized to the cytoplasm and plasma membrane. Yet Cx50 W4R was only

Table 2. Functional prediction on c.10T>A (p.W4R) missense variant in *GJA8*.

Mutation	Reference	Exon No.	MutationTaster	PolyPhen-2	SIFT
c.10T>A	NM_005267	2	Disease-causing	1.000, P	0.000, D

P, probably damaging; D, damaging.

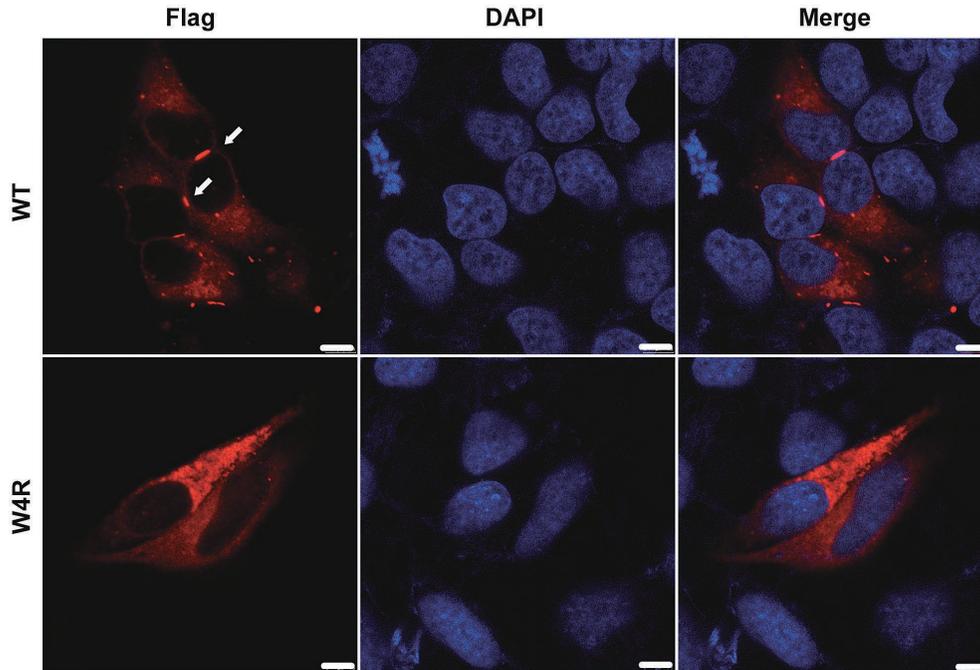


Fig. 2. Impaired gap junction formation with Cx50 p.W4R protein. Immunofluorescent imaging of Cx50 WT-Flag and Cx50 W4R-Flag in transiently transfected HeLa cells. Cells were immunostained with anti-Flag monoclonal antibody (red). DAPI shows nuclear DNA staining (blue). The arrows show gap junction plaques. Bar = 75 μ m.

expressed in the cytoplasm (Fig. 2) and was not transported to the membrane. Moreover, Cx50 WT formed gap junction plaques between adjacent cells (Fig. 2, shown as arrows) while the gap junction in Cx50 W4R-Flag was hardly detected. Our results suggest that the W-to-R substitution at codon 4 may affect Cx50 trafficking to the plasma membrane to form a gap junction.

Discussion

Congenital cataract is one of the most common ocular disorders leading to visual impairment and blindness in children worldwide. NGS-based genetic diagnosis is a potential technique to clarify the marked heterogeneity on genotype and phenotype of congenital cataract, for it can be achieved in approximately 70% of familial or sporadic non-syndromal cases (Ma et al. 2016). In this study, targeted exome sequencing was successfully used to identify a c.10T>A mutation of *GJA8* gene in our pedigree. The mutation co-segregated in patients and is absent in 200 unrelated normal individuals as well as dbSNP database, indicating that the mutation is responsible for this cataract phenotype, rather than a rare polymorphism in the Chinese

population.

Gap junction channel is permeable to ions and small solutes, and it plays important roles in a series of cellular processes, such as volume regulation (Shakespeare et al. 2009), the influx/efflux of ATP (Lohman and Isakson 2014), and cell death (Kalvelyte et al. 2003; Chi et al. 2012). The eye lens is an avascular structure, and it relies largely on intercellular communication mediated by gap junctions connexin 43 (Cx43), connexin 46 (Cx46), and Cx50 to support lens homeostasis and transparency (White 2002). GJA8 (Cx50) is a lens gap junction protein expressed in the lens, which modulates diffusion of molecules between lens fiber cells.

Connexins include an N-amino terminus, four transmembrane domains linked by a single cytoplasmic loop and two extracellular domains, and a carboxyl terminus. So far, 26 mutations in the different domains of Cx50 have been identified to contribute to human inherited cataracts according to 1000 Genome Project (<http://www.internationalgenome.org/>). A previous study revealed that knockout of Cx50 in mice causes lens growth deficiency, microphthalmia, and mild nuclear pulverulent cataracts (Gerido et al.

2003). Cx50 mutant mice showed developed microphthalmia with dense cataracts for unable to form functional channels (Chang et al. 2002). In the present study, we found the c.10T>A mutation that causes the W-to-R substitution, located in the N-amino terminus. The W residue at codon 4 is highly conservative among species, and the mutation is predicted to impair Cx50 protein function. Moreover, the N-terminal domain of connexin was reported to influence transjunctional voltage-dependent gating (Verselis et al. 1994; Oh et al. 2000), channel conductance (Musa et al. 2004), and permeability (Dong et al. 2006). A reported cataract pathogenic mutant CX50 R23T impaired trafficking and formation of gap junction, which might be caused by the replacement of the positively charged amino acid R with an uncharged, polar amino acid Threonine (T) (Thomas et al. 2008). Accordingly, the Cx50 W4R might lead to the uncharged, polar amino acid W substituted by a positively charged amino acid R as well. Likewise, the Cx50 protein distribution results indicated that Cx50 W4R affects subcellular distribution and trafficking of Cx50 protein to the plasma membrane, which in turn cannot organize into normal hemichannel. Thus, dysfunctional gap junction might be a possible mechanism by which the identified Cx50 mutant could lead to cataract.

In summary, we identified a heterozygous c.10T>A (p.W4R) mutation in *GJA8* as the causative mutation in our Chinese congenital cataract pedigree. Distinct Cx50 localization and abnormal gap junction formation were caused by the mutant protein in transfected HeLa cells, which may account for the molecular basis and underlying mechanisms of cataract in this family.

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Author Contributions

J.S. and S.J. conceived and designed the experiments; L.Z. and Y.L. performed the experiments; Y.Z. and H.Z. got authority from patients and controls, and helped to collect the peripheral blood samples; L.Z. wrote the paper.

Conflict of Interest

The authors declare no conflict of interest.

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