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Original Article

Targeted exome sequencing identified a novel *GJA3* gene missense mutation causes autosomal dominant congenital cataract in a large Chinese family

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Abstract: Objectives: Congenital cataract is the leading cause of visual impairment or blindness in children. The purpose of this study is to screen for pathogenic mutations in a five-generation Chinese family affected with autosomal dominant congenital cataract. Methods: A five-generation Chinese family born with congenital cataract was investigated. A specific hereditary eye disease enrichment panel based on targeted exome capture technology was performed in the proband, and Sanger sequencing was then conducted in other 23 family members and 100 normal controls. Bioinformatics analysis was used to determine the possibility of the changes affect the phenotype. Results: A novel heterozygous missense mutation (c. 584C>T) was identified in the exon 2 of the gap junction protein-alpha 3 (*GJA3*) gene, which resulted in the substitution of a serine with a phenylalanine (p.S195F). This mutation cosegregated with twelve affected members of the family, but unidentified in the unaffected family members and normal controls. The p.S195F mutation was found in the second extracellular loop domain of GJA3 protein. Bioinformatics analysis suggested that the p.S195F mutation was predicted to be disease causing, with the change of protein structure from monomer to homer-12-mer. Conclusions: This is the first report of the novel c. 584C>T (p.S195F) missense mutation in the *GJA3* gene was the genetic cause of congenital cataract. The results also suggested that the homer-12-mer was the most common mutation structure, and the extracellular loop was the mutation hotspot of GJA3 protein.

Keywords: Congenital cataract, connexin 46, GJA3

Introduction

Congenital cataract is known as a major causation of children impairment of vision or blindness. Due to the major characteristics of congenital cataract, lens opacities and childhood onset, congenital cataract has negative influence on individual visual development and lead to form deprivation amblyopia [1]. The incidence of congenital cataracts is 6.31/100,000 [2], and would be much lower in industrialized countries compared with poor areas among the world [3]. The mode of congenital cataract hereditary includes autosomal dominant, autosomal recessive, or X-linked, among which autosomal dominant is the most common [2].

Autosomal dominant congenital cataracts (ADCC) as congenital cataracts are genetically heterogeneous, which is known that the highly variable morphologies of cataracts within some families. These clinical features suggest that the same mutation in a single gene can lead to different phenotypes [4, 5]. In previous studies, ADCC have been linked to the mutations in at least 23 specific causative genes, which encode the main cytoplasmic proteins of human lens, including crystallin genes (*CRYAA*, *CRYAB*, *CRYBA1*, *CRYBA2*, *CRYBB1*, *CRYBB2*, *CRYGB*, *CRYGC*, *CRYGD*, and *CRYGS*), connexin genes (*GJA3* and *GJA8*), MIP, BFSP2, PITX3, MAF, and HSF4 genes [4].

In this study, we conducted exome sequencing of widely reported congenital cataract pathogenic genes to identify the mutation in a Chinese five-generation pedigree. A novel missense mutation (c.584C>T) was detected in the

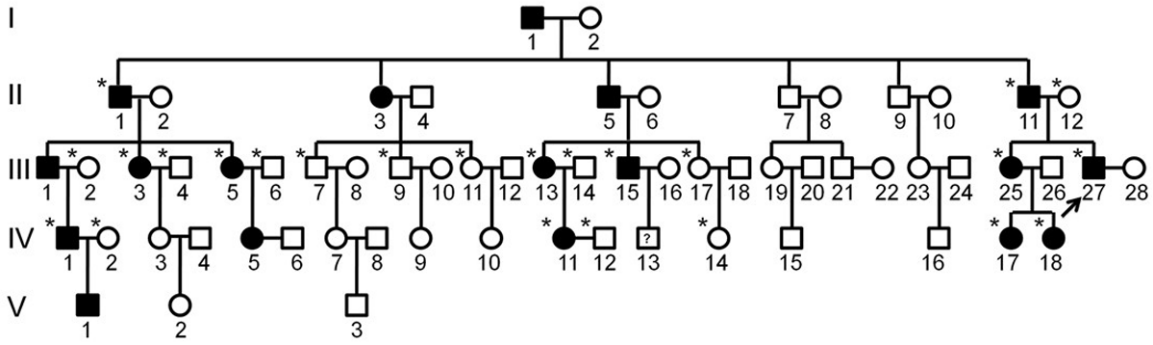

Figure 1. Pedigree of five-generation family with autosomal dominant congenital cataract. Squares and circles indicate males and females. Black and white symbols represent affected cases and unaffected individuals. Asterisks indicate family members who attend this study. Arrow indicates the proband.

Table 1. Primer sequences for *GJA3* gene amplification and sequencing

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)
GJA3-E1A	CGGTGTTCATGAGCATTTTC	CCTGCTTGAGCTTCTTCCAG	743
GJA3-E1B	ACGGTGGACTGCTTCATCTC	GCACTTTGGTTTTGGTTTCTAA	810

Table 2. Variantsin *GJA3* gene have been found in all family members

Family Individuals	Gender	Cataract	*GJA3 c.584C>T*	*GJA3* c. 895C>A
II-1	Male	Yes	+	-
II-11	Male	Yes	+	+
II-12	Female	No	-	-
III-2	Female	No	-	-
III-3	Female	Yes	+	-
III-4	Male	No	-	-
III-5	Female	Yes	+	-
III-6	Male	No	-	-
III-7	Male	No	-	-
III-9	Male	No	-	-
III-11	Female	No	-	-
III-13	Female	Yes	+	-
III-14	Male	No	-	-
III-15	Male	Yes	+	-
III-17	Female	No	-	-
III-25	Female	Yes	+	-
III-27	Male	Yes	+	+
IV-1	Male	Yes	+	-
IV-2	Female	No	-	-
IV-11	Female	Yes	+	-
IV-12	Female	No	-	-
IV-14	Male	No	-	-
IV-17	Female	Yes	+	-
IV-18	Female	Yes	+	-

GJA3 gene, with resultant Phe substitution for the highly conserved Ser at codon 195 (p. S195F). This mutation was unidentified in the unaffected family members and the 100 normal controls, indicating that it may be a pathogenic mutation for ADCC in this family.

Materials and methods

Study subjects and clinical evaluation

Owning to seeking the gene therapy for the next generation of the proband (III:27), a five-generation, sixty three-member Chinese Huang family with autosomal dominant cataract from Liaoning province, China, was recruited from the Peking University Third Hospital (**Figure 1**). There were twelve patients in the three generations (II:1, II:11, III:3, III:5, III:13, III:15, III:25, III:27, IV:1, IV:11, IV:17-18) of the pedigree with congenital cataract. All subjects were intraocular lens, except a one-year child, and underwent detailed ophthalmic examinations, including visual acuity, slit lamp examination, fundus examination with the dilated pupils, and intraocular pressure measurement. One hundred matched healthy were randomly recruited to serve as normal controls. Informed consent was obtained from all individuals. All procedures used in this study conformed to the tenets of the Declaration of Helsink and

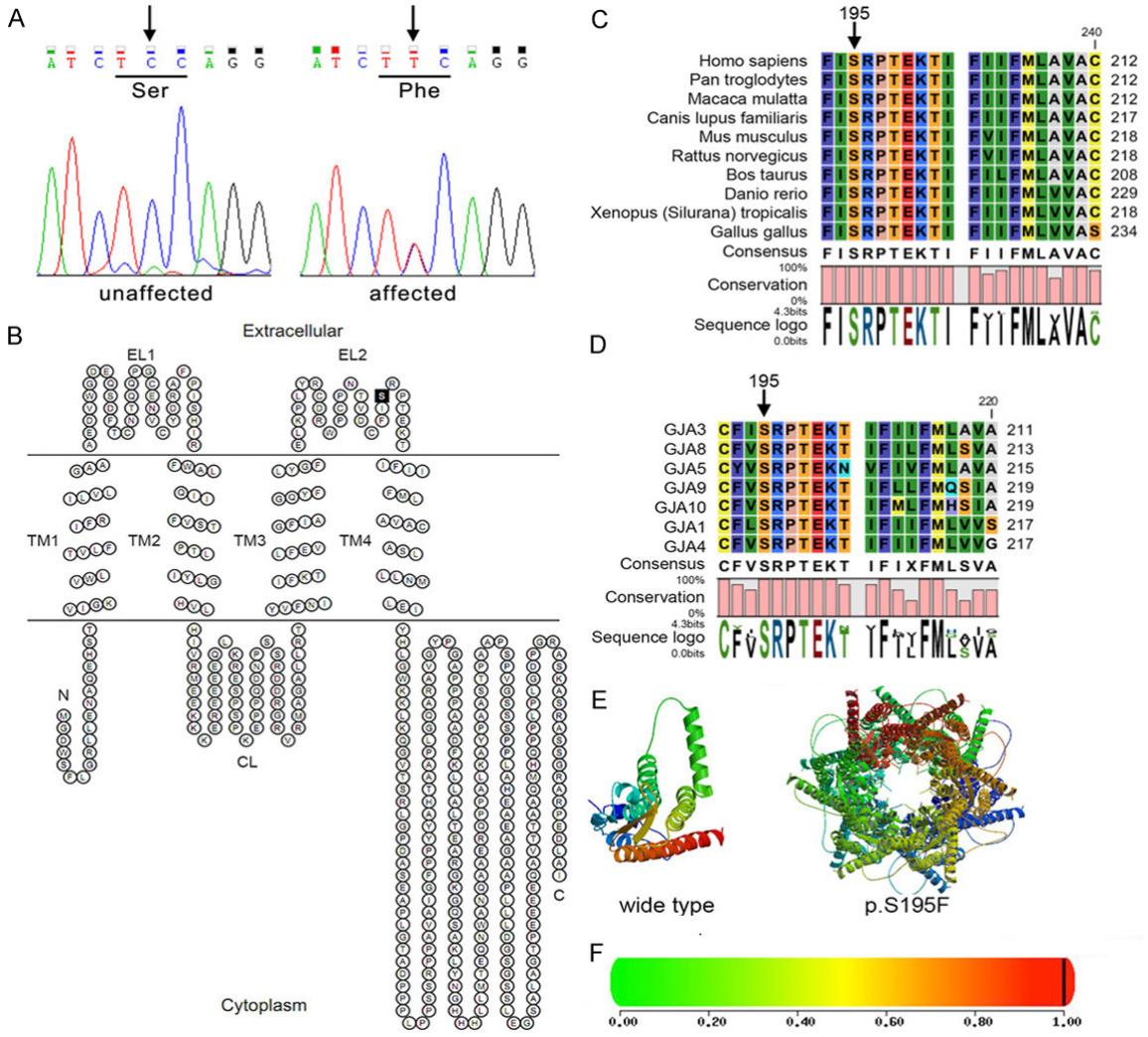

Figure 2. Mutational analysis. A. DNA sequence of the GJA3 gene in affected and unaffected individuals in the autosomal-dominant congenital cataract Chinese family. A heterozygous change is observed at position 584 (C>T) as a C>T double peak (indicated by a black arrow), resulting in the substitution of Serine by Phenylalanine (S195F) of connexin 46 in the affected individuals. B. The membrane topological structure of connexin 46 was generated based on TMpred using TOPO2. p.S195F mutation (indicated by solid black square) is located in the second extracellular loop. N, NH2-terminus; TM1, first transmembrane domain; TM2, second transmembrane domain; TM3, third transmembrane domain; TM4, fourth transmembrane domain; EL1, first extracellular loop domain; EL2, second extracellular loop domain; CL, cytoplasmic loop domain; C, COOH-terminus. C. Serine at position 195 of connexin 46 is highly conserved in different species (indicated by an arrow). D. Serine at position 195 is highly conserved in different human gap junction proteins (indicated by an arrow). E. Modeled structure of connexin 46. The p.S195F mutation tends to be in the form of homo-12-mer, which was different with the monomer structure for the wide type protein. F. The p.S195F mutation is predicted to be causative by PolyPhen-2, with a score of 1.00.

approved by Peking University Third Hospital Medical Ethics Committee.

Genomic DNA sample preparation

Five milliliters of venous blood was collected from participating family members and controls in BD Vacutainers (BD, San Jose, CA) containing EDTA. Genomic DNA was extracted using QIAamp DNA Blood Mini Kits (QIAGEN Science, Germantown, MD), and was quantified by Nanodrop 2000 (Thermo Scientific, Rockford, IL).

Mutation screening

A specific hereditary eye disease enrichment panel based on targeted exome capture technology was used to capture the mutation of gene in the proband (MyGenostics GenCap

Table 3. The summary of mutations of congenital cataract related with GJA3 gene

Mutation	Amino Acid Changes	Location	Cataract Type	Family Origin	3D Structural Model of Protein	Reference
c.5G>A	p.G2D	NH2-terminus	Zonular pulverulent and posterior polar	Chinese	Mono	[13]
c.7G>T	p.D3Y	NH2-terminus	Zonular pulverulent	Hispanic Central American	Mono	[14]
c.32T>C	p.L11S	NH2-terminus	Ant-egg	Danish	Homo-12-mer	[15]
c.56C>T	p.T19M	NH2-terminus	Posterior polar	Indian	Homo-12-mer	[16]
c.82G>A	p.V28M	TM1	Variable	Indian	Homo-12-mer	[17]
c.96C>A	p.F32L	TM1	Nuclear pulverulent	Chinese	Mono	[18]
c.98G>T	p.R33L	TM1	Embryonal nuclear granular	Indian	Homo-12-mer	[19]
c.130G>A	p.V44M	EL1	Bilateral nuclear	Chinese	Homo-12-mer	[20]
c.134G>C	p.W45S	EL1	Bilateral nuclear	Chinese	Homo-12-mer	[21]
c. 139G>A	p.D47N	EL1	Nuclear	Chinese	Homo-12-mer	[22, 23]
c.163A>G	p.N55D	EL1	Central nuclear opacity	Chinese	Homo-12-mer	[24]
c.176C>T	p.P59L	EL1	Nuclear punctate	American	Homo-12-mer	[25]
c.188A>G	p.N63S	EL1	Zonularpulverulent	Caucasian	Homo-12-mer	[26]
c.226C>G	p.R76G	EL1	Total	Indian	Homo-12-mer	[17]
c.227G>A	p.R76H	EL1	Pulverulent	Australian	Homo-12-mer	[17, 27]
c.260C>T	p.T87M	TM2	Pearl box	Indian	Mono	[17]
c.427G>A	p.G143R	CL	Coppock-like cataract	Chinese	Mono	[28]
c.428G>A	p.G143E	CL	Bilateral nuclear cataract	Chinese	Mono	[29]
c.559C>T	p.P187S	EL2	Zonularpulverulent	Chinese	Homo-12-mer	[30]
c.560C>T	p.P187L	EL2	Zonularpulverulent	Caucasian	Homo-12-mer	[31]
c.563A>C	p.N188T	EL2	Nuclear pulverulent	Chinese	Mono	[18]
c.563A>T	p.N188I	EL2	Zonularpulverulent	Chinese	Homo-12-mer	[32]
c.584C>T	p.S195F	EL2	Unknow	Chinese	Homo-12-mer	This study
c.589C>T	p.P197S	EL2	Posterior subcapsular	Indian	Homo-12-mer	[33]
c.616T>A	p.F206I	TM4	Bilateral nuclear	Chinese	Homo-12-mer	[34]
c.1137insC	p.S380fs	COOH-terminus	Zonularpulverulent	Caucasian	Homo-12-mer	[35]
c.1361insC	p.A397fs	COOH-terminus	Coralliform	Chinese	Homo-12-mer	[36]

CL, cytoplasmic loop; EL1, first extracellular loop; EL2, second extracellular loop; TM1, first transmembrane domain; TM2, second transmembrane domain; TM4, fourth transmembrane domain.

Enrichment technologies, Beijing, China). Briefly, three micrograms of genomic DNA of the proband was used to prepare the DNA libraries according to Illumina's protocol. The sequencing libraries were enriched for the target region related genes using the MyGenostics Target Region Enrichment protocol. The captured libraries were sequenced using Illumina HiSeq 2500 Sequencer. After HiSeq 2500 re-sequencing, raw sequencing reads were first filtered by the Solexa QA package and then by the cut adapt program (http://code.google.com/p/cutadapt/). High quality reads were aligned to the reference genome sequence (hg19). The clean read sequences were mapped by SOAP aligner (http://soap.genomics.org.cn/soapaligner.html) and Burrows-Wheeler Aligner (BWA) (http://bio-bwa.sourceforge.net/bwa.shtml). The SNPs and Indels were identified by the GATK program (http://www.broadinstitute.org/gsa/wiki/index.php/Home_Page) and the SOAPsnp program (http://soap.genomics.org.cn/soapsnp.html). Identified SNPs and InDels were annotated using the Exome-assistant program (http://122.228.158.106/exomeassistant).

A total of 371 disease genes in the panel are associated with hereditary eye disease genes, and 164 genes are considered to have a relationship with cataract and systemic syndrome with cataract, including *ABHD12, ADAM9, ADAMTS10, ADAMTSL4, AGK, AGPS, AKR1E2, ALDH18A1, APOE, APOPT1, ATOH7, B3GALTL, BCOR, BEST1, BFSP1, BFSP2, CAPN15, CASR, CBS, CHMP4B, CNBP, COL11A1, COL18A1, COL2A1, COL4A1, COL7A1, CRYAA, CRYAB, CRYBA1, CRYBA2, CRYBA4, CRYBB1, CRYBB2, CRYBB3, CRYGA, CRYGB, CRYGC, CRYGD, CRYGS, CTDP1, CTNND2, CYP27A1, CYP51A1, DHCR7, DMPK, EPG5, EPHA2, ERCC2, ERCC3, ERCC6, ERCC8, ESR1, EYA1, EZR, FAM126A,*

FBN1, FKRP, FKTN, FOXC1, FOXD3, FOXE3, FTL, FTO, FYCO1, FZD4, GALK1, GALT, GCNT2, GFER, GJA1, GJA3, GJA8, GNPAT, GSTM1, GSTT1, HMX1, HSF4, IDO1, IFNGR1, ITM2B, JAM3, KCNJ13, KLC1, LARGE, LCA5, LCT, LIM2, LMX1B, LRP5, LRRD1, LTBP2, MAF, MAN2B1, MFSD6L, MIP, MIPEP, MIR5689, GCNT2, MTHFR, MVK, MYH9, NAA16, NAT2, NBPF10, NDP, NF2, NHS, NOG, OAT, OCRL, OPA3, PAX6, PEX10, PEX11B, PEX12, PEX13, PEX14, PEX16, PEX19, PEX2, PEX26, PEX3, PEX5L, PEX6, PEX7, PITX2, PITX3, POMGNT1, POMT1, POMT2, PVRL3, PXDN, RAB18, RAB3GAP1, RAB3GAP2, RECQL4, RNLS, SC5D, SEC23A, SIL1, SIX5, SIX6, SLC16A12, SLC25A15, SLC2A1, SLC33A1, SLC38A1, SLC7A14, SOX2, SPRYD4, SRD5A3, SREBF2, TDRD7, TFAP2A, TGFB3, TMEM114, TMEM70, UNC45B, VIM, VLDLR, VSX2, WFS1, WRN, XRCC1.

DNA samples of the other family members and 100 ethnically matched normal control were used to validate all likely pathogenic variants by using conventional Sanger sequencing. The coding regions of the candidate gene, the *GJA3* gene, were amplified by polymerase chain reaction (PCR) with the primers listed in **Table 1** and screened for mutations on both strands using bidirectional sequencing. The sequencing results were analyzed using Chromas 2.33 and compared with the reference sequences in the NCBI database.

Bioinformatics analysis

Based on TMpred, TOPO2 was used to generate the membrane topological structure of the GJA3 gene-encoded Cx43 protein (http://www.sacs.ucsf.edu/TOPO2/). Multiple sequence alignment was performed using the CLC Free Workbench 6.9 software (CLC bio, Aarhus, Denmark). The possible impact of an amino acid substitution on the structure and function of the protein was predicted by PROVEAN (Protein Variation Effect Analyzer, http://provean.jcvi.org/index.php) [6, 7], Polymorphism Phenotyping version 2 (http://genetics.bwh.harvard.edu/pph2/) [8] and Mutation Taster (http://www.mutationtaster.org) [9]. The predictions of three-dimensional (3D) structures of wild type, mutant proteins and the past reported mutations were conducted by the online SWISS-MODEL tool (http://swissmodel.expasy.org/) [10-12].

Results

Clinical findings

For the family history of congenital cataract, the proband (III:27) argued to be identified the specific mutation gene of the congenital cataract in order to conduct gene therapies of the next generation. We have identified 24 family members, in which 12 were patients (**Figure 1**). After reviewing the past history and the details of surgery, all patients were born with bilateral cataract and the existing patients have all undergone cataract surgery between the ages of 1 and 68 years, except the youngest one-year child without the IOL implantation. To data, all individuals did not have other clinically oculopathy correlated systemic diseases.

Mutation analysis

The 371 hereditary eye disease related genes were captured and sequenced by next generation sequencing on the proband. Overall, 99.66% of the targeted disease gene regions were sequenced. The fraction of target covered with at least 4X, 10X, and 20X were 99.57%, 99.13%, and 98.14%, respectively. In total, 172 coding variants were identified in the proband's sample through the bioinformatic analysis, including 61 non-synonymous, 1 deletion, and 1 insertion variant. The variants were filtered out if they showed up in the 1000 genome database and the dbSNP database. After the bioinformatic filtration, novel compound heterozygosity for c.584C>T and c.895C>A variants in the *GJA3* gene were found, and the c.584C>T variant was identified as the predicted homozygous pathogenic mutation.

The two variants in the *GJA3* gene identified using next-generation sequencing were further confirmed by Sanger sequencing validation and segregation analysis in the proband and other 23 recruited family members. The results revealed that the heterozygous *GJA3* c.584C>T mutation was detected in all 12 affected individuals, but neither present in unaffected family members (**Figure 2A**; **Table 2**). However, only the proband (III:27) and his father (II:11) were detected another heterozygous *GJA3* c.895C>A variant, which demonstrated that the c.584C>T variant was not cosegregated with the disease

phenotype in this family (**Table 2**). The *GJA3* c.584C>T mutation, as the disease-causing variant of this family, was not detected in 100 unrelated normal controls.

Bioinformatics analysis for GJA3 c.584C>T mutation

The *GJA3* c.584C>T mutation identified in this family is a missense mutation and leads to an amino acid change from Ser to Phe at codon 195 (p.S195F) in the *GJA3* gene-encoded human connexin protein connexin 46. The p.S195F missense mutation is located in the second extracellular loop domain of connexin 46 using the topology prediction servers TMpred and TOPO2 (**Figure 2B**). Serine (S) is a highly conserved amino acid in vertebrate species (**Figure 2C**), and also among different types of the human connexin family (**Figure 2D**).

According to the prediction of 3D structural model of connexin 46, the p.S195F mutation can hardly be stable as a monomer, but tends to be in the form of homo-12-mer, which was different with the wide type (**Figure 2E**).

This p.S195F mutation was predicted to be "disease causing" by Mutation Taster analysis, "deleterious" by PROVEAN, "damaging" by PolyPhen-2 analysis with a score of 1.000 (sensitivity: 0.000; specificity: 1.000) (**Figure 2F**).

Mutation spectrum in the GJA3 gene associated with autosomal dominant congenital cataracts

The *GJA3* gene is comprised of two exons, and the protein-coding regions is 1307 bp in the exon 2. Twenty-seven mutations in the *GJA3* gene associated with ADCC had been summarized in **Table 3**, in which 27 were previously reported and one was identified in this study. Among these mutations, 25 mutations cause the amino-acid substitution of connexin 46 protein, and 2 mutations are insertion mutation in the COOH-terminus. The most mutations tend to be in the form of homo-12-mer protein by the prediction of 3D structural model of protein.

Discussion

In our study, targeted exome sequencing were used to screen 164 known congenital cataract genes, and a homozygous mutation of the *GJA3* gene was co-segregated in this family. The c.584C>T variant is a missense mutation in the second exon of *GJA3* gene and leads to the substitution of Ser to Phe at amino acid 195 (p.S195F). Moreover, this variant was not detected in other 100 controls, suggesting that it is a disease causing mutation rather than a polymorphism. The PROVEAN, Mutation Taster, and PolyPhen-2 prediction tools all suggested that this mutation affects protein function and is a disease causing mutation. Overall, these data support that the congenital cataract in this family is caused by the homozygous *GJA3* c.584C>T mutation.

The human *GJA3* gene, mapped on 13q12.11, was first reported to cause ADCC by Willecke *et al* in 1990 [37]. This *GJA3* gene includes two exons and a 435-amino acid protein, known as connexin 46, is only encoded by the exon 2.

Connexin 46 contains four transmembrane domains (TM), two extracellular loops (EL), an intracellular loop (CL), NH2-terminus, and COOH-terminus in the cytoplasm, among which two extracellular loops are the most conserved regions and play a crucial role in regulating hemichannels docking [24, 38]. This protein, similar as other connexins, functioned as a kind of intercellular channel for voltage and chemical gating [39], but only connexin 46 has been reported to be associated with calcium influx and play an important role on the function and development of lens according to various animal studies [40]. After knocking out *Gja3* gene, mice present with large amount of calcium influx and dramatically glutathione decreasing in the nucleus, leading to the crystallins cleavage and insoluble complex aggregation, eventually, developed into cataract [41-43]. It is obvious that connexin 46 is closely related with lens transparency maintaining. However, the exact mechanism of mutated connexin 46 in cataract is still unknown.

To our best, there 27 mutations in human *GJA3* gene have been reported as pathogenic mutations for congenital cataract. Among these mutations, four were located in NH2-terminus, five in the TMs, two in the CL, two in the COOH-terminus, and interestingly, half in the ELs, may indicate the founder effect or mutation hotspot of the ELs.

In the current study, the homozygous missense mutation p.S195F in our patients lied on the

EL2 of connexin 46, which has been reported to play a key role in regulating intercellular docking via cysteine-rich domain [44, 45]. The serine at position 195 is located in highly conserved region of various species and different human connexins. Due to the replacement hydrophobic phenylalanine of polar neutral serine, the polarity of second extracellular domain-changed, which may not only disrupts the polarity of ions channels, but also changes the secondary structure of connexin 46, with the resultant of gap junction dysfunction. The full score of PolyPhen-2 indicated the highly pathogenic potential of the mutation. And the 3D prediction of other mutations of *GJA3* gene also suggested the distinct structure altering of p. S195F. In addition, homo-12-mer was the most common format of the abnormal protein. Compared to the wide type, the homo-12-mer aggregation largely increases molecular weight of connexin 46, consequently, following with abnormal aggregation of the connexins.

The detecting strategy to reveal genetic mutations is notable in this study. For the size of the family and the ADCC related genes were so large, uniting targeted exome sequencing to identify the proband and Sanger sequencing for the rest could be much more efficient and economical.

However, the details of phenotype associated with the mutation site could not be known, as all patients in the family had cataract extraction performed. Since the youngest patient is less than 2 years of age, it suggested that the mutations cause early onset, and even at birth. The past records of surgery could contribute to the specific diagnosis to some degree, and we will further follow up the family to identify the exact phenotype. But it is hardly to verify whether the lens opacities of all affected members were similar and whether the opacity was progressive.

Conclusion

In summary, we present a novel missense mutation c.584C>T (p.S195F) mutation of *GJA3* gene from an autosomal dominant congenital cataract family. These findings expand the mutation spectrum of GJA3 in terms of congenital cataracts and also provide an opportunity for the proband received gene therapy trying to produce unaffected next generation.

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Disclosure of conflict of interest

None.

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