A novel donor splice-site mutation of major intrinsic protein gene associated with congenital cataract in a Chinese family

Lu Zeng,[1] Wenqiang Liu,[1] Wenguo Feng,[2] Xing Wang,[1] Hui Dang,[2] Luna Gao,[1] Jing Yao,[3] Xianqin Zhang[1]

(The first four authors contributed equally to this work)

[1]*Key Laboratory of Molecular Biophysics, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China;* [2]*Jinan Second People's Hospital,Jinan, China;* [3]*College of Life Science, Wuhan University, Wuhan, China*

Purpose: To identify the disease-causing gene in a Chinese family with autosomal dominant congenital cataract.
Methods: Clinical and ophthalmologic examinations were performed on all members of a Chinese family with congenital cataract. Nine genes associated with congenital cataract were screened using direct DNA sequencing. Mutations were confirmed using restriction fragment length polymorphism (RFLP) analysis. The mutated major intrinsic protein (*MIP*) minigene, which carries the disease-causing splice-site mutation, and the wild-type (WT) *MIP* minigene were constructed using the pcDNA3.1 expression vector. Wild-type and mutant *MIP* minigene constructs were transiently transfected into HeLa cells. After 48 h of incubation at 37 °C, total RNA isolation and reverse transcription (RT)–PCR analysis were performed, and PCR products were separated and confirmed with sequencing.
Results: Direct DNA sequence analysis identified a novel splice-site mutation in intron 3 (c.606+1 G>A) of the *MIP* gene. To investigate the manner in which the splice donor mutation could affect mRNA splicing, WT and mutant *MIP* minigenes were inserted in the pcDNA3.1 (+) vector. Constructs were transfected into HeLa cells. RT–PCR analysis showed that the donor splice site mutation led to deletion of exon 3 in the mRNA encoded by the *MIP* gene.
Conclusions: The present study identified a novel donor splice-site mutation (c.606+1G>A) in the *MIP* gene in a Chinese family with congenital cataract. In vitro RT–PCR analysis showed that this splice-site mutation resulted in the deletion of exon 3 from mRNA encoded by the *MIP* gene. This is the first report to show that donor splice-site mutation in *MIP* gene can cause autosomal dominant congenital cataract.

Congenital cataracts are defined as an opacification of the eye lens appearing at birth or shortly thereafter. They are the main cause of blindness in children all over the world. About one third of infants with congenital cataracts will lose their sight, and 50% of these cases are inherited. Most congenital cataracts are inherited as autosomal dominant traits [1-4]. Congenital cataracts are classified into several subtypes: whole lens, nuclear, lamellar, cortical, polar, sutural, pulverulent, cerulean, and coralliform [5].

To date, more than 40 genetic loci have been linked to congenital cataracts, and at least 26 genes have been cloned and sequenced. These numbers are continually increasing. Among these, genes encoding crystallins and genes encoding membrane transport and channel proteins are the most common genetic causes of nuclear cataracts [5-7].

In this study, a Chinese family with nuclear cataracts was identified and characterized, and mutation screening was performed on genes associated with nuclear cataracts using direct DNA sequencing. A novel donor splice site mutation was identified in intron 3 (c.606+1 G>A) of the major intrinsic protein (*MIP*) gene.

METHODS

Clinical data and isolation of human genomic deoxyribonucleic acid: The study participants (8 affected individuals and 7 unaffected individuals) were identified and enrolled at Jinan Second People's Hospital. Clinical and ophthalmological examinations were performed by ophthalmologists in the hospital. Written informed consent was obtained from the study subjects. This study was approved by the ethics committee of Huazhong University of Science and Technology. Peripheral blood was withdrawn into EDTA2K tubes from the participants. Human genomic DNA was isolated from the whole blood samples using a DNA Isolation Kit for Mammalian Blood (Roche Diagnostic, Indianapolis, IN).

Mutation screening: Nine candidate genes were selected for the mutation screening. These genes included γC-crystallin (*CRYGC*), γD-crystallin (*CRYGD*), connexin 46 (*Cx46*),connexin 50 (*Cx50*),βA1-crystallin (*CRYBA1*), βB1-crystallin (*CRYBB1*), βB2-crystallin (*CRYBB2*),

Correspondence to: Xianqin Zhang, College of Life Science and Technology, Huazhong University of Science and Technology, 1037 Luoyu Road, Wuhan, China, Phone: +86 87792649; email: xqzhang04@hust.edu.cn

βA4-crystallin (*CRYBA4*) and *MIP*. All exons of candidate genes, including exon-intron boundaries, were PCR-amplified and sequenced. PCR was performed in 25 µl of standard PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 1 unit of Taq DNA polymerase, and 25 ng of human genomic DNA. The amplification program was one cycle of 3 min of denaturation at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 65 °C, 1 min at 72 °C, and one 7 min extension step at 72 °C. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). They were sequenced with forward and reverse primers. DNA sequencing analysis was performed using a BigDye Terminator Cycle Sequencing v3.1 kit and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Restriction fragment length polymorphism analysis: The mutation (c.606+1 G>A) produced a BtsC I restriction enzyme site. Flanking sequences of intron 3 amplified with PCR from all members of the family and 100 unrelated normal individuals using primers: MIP-exon 3-(MIP-exon 3 forward: 5′-GTG CAG TAG GGG GGT GTC TCA GTT-3′; MIP-exon 3 reverse: 5′-ATC TTG CCC CTC TCC CTT CAC T-3′). PCR products were digested overnight with BtsC I at 55 °C and separated on 3% agarose gels by electrophoresis.

Plasmid and minigene constructs: Mutated (MUT) *MIP* minigenes, which carry the disease-causing splice-site mutation, and wild-type (WT) *MIP* minigenes were constructed using a pcDNA3.1 expression vector. These minigenes consisted of intron 1, exon 2, intron 2, exon 3, intron 3, and partial exon 4 of the *MIP* gene. These were amplified separately using primers (3.1*MIP* forward: 5′-TAT AGA ATT CCG GGG AAG TCT TGA GGA GGT AAC-3′; 3.1*MIP* reverse: 5′-TAA TCT CGA GCG GGG GAA GAG AAG AAA GTC GTA C-3′) from affected and unaffected family members. The PCR products were then cloned into the pcDNA3.1 (+) expression vector to generate the pcDNA3.1-MIP-WT and pcDNA3.1-MIP-MUT minigene expression vector.

Transcription analysis of the wild-type and mutant mini-intronic plasmid gene: To determine whether the *MIP* c.606+1 G>A mutation could influence the splicing of pre-mRNA, pcDNA3.1-MIP-WT, pcDNA3.1-MIP-MUT, and the control pcDNA3.1 (+) plasmid were transiently transfected into HeLa cells. Total RNA was extracted and analyzed using reverse transcription (RT)–PCR.

Cell culture and transient transfection: HeLa cells were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and cultured with 5% CO_2 at 37 °C. Then 4 µg of wild-type minigene constructs, mutant minigene constructs, and

control plasmid pcDNA3.1(+) were transiently transfected into HeLa cells with Lipofectamine 2000 (Invitrogen).

Reverse transcription polymerase chain reaction analysis: Total RNA was extracted from the HeLa cells 48 h after transfection, using TRIzol reagent, in accordance with standard procedures. RNA samples were treated using RQ1 RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's instructions. And 2 µg of these treated RNA samples were used for synthesis of cDNA in a 25 µl system with random primers and M-MLV Reverse Transcriptase Promega. Primers for second-strand synthesis included a forward primer (5′-TTG CAC CCT GCG GTG AGC GTG G-3′) and a reverse primer (5′-CGG GGG AAG AGA AGA AAG TCG TAC-3′). The PCR products were separated on 2% agarose gels, purified, and sequenced.

RESULTS

Clinical diagnostic findings: A four-generation Chinese family with autosomal dominant congenital cataract (ADCC) was identified (Figure 1). The proband was a 5-year-old girl (IV:1 in Figure 1). Slit-lamp examination showed nuclear cataracts in both eyes (Figure 2). Similar clinical features were detected in other affected family members.

Mutation analysis: Mutation screening of candidate genes was performed with direct DNA sequencing, a novel mutation (c.606+1 G>A) at the donor splice site of intron 3 of the *MIP* gene was identified in the family (Figure 3). Within the family, the mutation cosegregated with disease. All affected individuals carried the mutation, and all unaffected individuals did not. The mutation (c.606+1 G>A) creates a BtsCI restriction enzyme site. RFLP analysis using the BtsCI enzyme confirmed that the mutation cosegregated with disease in the family and that the mutation was not present in either the unaffected family members or in 100 normal Chinese controls.

Transcription analysis of the mutant mini-intronic plasmid gene: To determine whether the *MIP* c.606+1 G>A mutation could affect the splicing of mRNAs, pcDNA3.1-MIP-WT, pcDNA3.1-MIP-MUT, and the control pcDNA3.1 (+) plasmid were transiently transfected into HeLa cells. RNA was extracted and analyzed using RT–PCR and then separated on 2% agarose gels. RT–PCR of total RNA obtained from cells transfected with pcDNA3.1-MIP-WT produced a 317 bp band consistent with the correct splicing of mRNA, and a 236 bp band was observed in cells transfected with pcDNA3.1-MIP-MUT. No bands were detected in the cells transfected with pcDNA3.1 (+) control (Figure 4). The RT–PCR products were purified and sequenced, and sequencing analysis showed that the *MIP* donor splice-site mutation (c.606+1 G>A) caused the
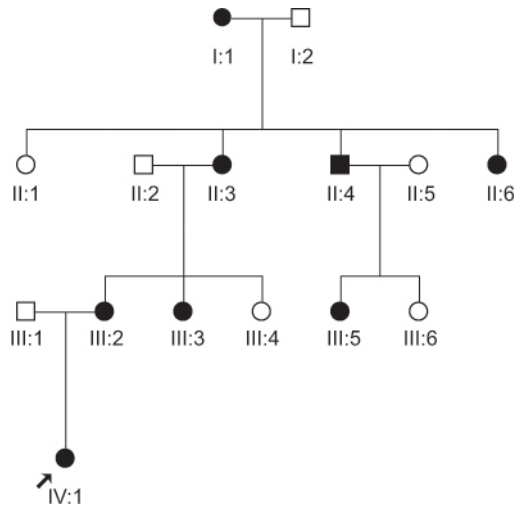
Figure 1. Pedigree of a four-generation Chinese family with autosomal dominant congenital cataract (ADCC). The affected male and female individuals are indicated with filled squares and circles, respectively. Normal individuals are shown as empty symbols. The proband is indicated with an arrow.

deletion of exon 3 of the *MIP* gene during mRNA splicing (Figure 5).

DISCUSSION

In the present study, a novel splice donor site mutation was identified in intron 3 (c.606+1 G>A) of the *MIP* gene. This is the first report to show that donor splice-site mutation in the *MIP* gene can cause autosomal dominant congenital cataract. The mutation was found to cosegregate with the disease phenotype in the family and was not present in unaffected family members or in 100 normal Chinese controls.

The *MIP* gene encoded the Aquaporin0 (AQP0) protein. Aquaporins (AQPs) are integral membrane proteins. They are widespread transmembrane channels that facilitate the permeation of water and other small molecules across the cell membrane [8]. Since Peter Agre identified the water channel AQP1 in 1992, 13 isoforms of AQPs has been identified in

mammals; these AQPs are expressed in various tissues [9,10]. Among these isoforms, AQP0 and AQP1 are expressed in the lens and play an important role in keeping it transparent. AQP0 is expressed in lens fibers, and AQP1 is abundant in lens epithelial cells [11].

AQP0, also known as MIP, is a 28 kDa protein with 263 amino acids. It is inserted in the plasma membrane and is common in membrane proteins [11]. There are six transmembrane domains and intracellular NH_2- and COOH terminals (residue 220 to 263) in AQP0, and it assembles as a tetramer with four water pores. Each monomer is a water channel and can function independently [12]. AQP0 is a major permeability pathway of water in lens fibers [13]. Because there is no vasculature in the lens (including its center), it relies heavily on this conveyance system to maintain the normal flow of water across the cells and to keep the lens transparent and homeostatic [14]. Mutations in *MIP* in humans, wild-type
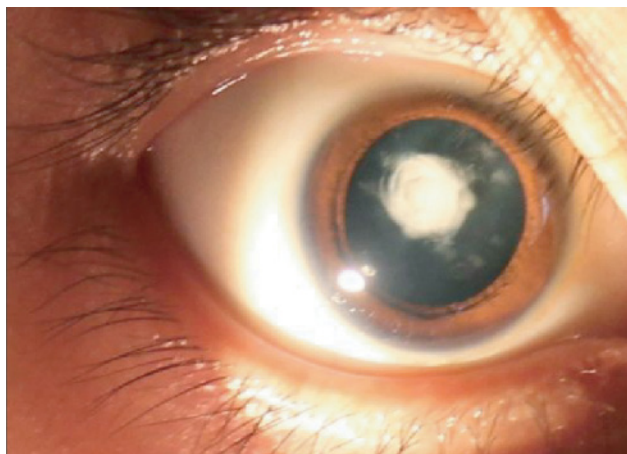

Figure 2. Photograph of the right eye of one affected individual (III:3 in Figure 1) in the family with autosomal dominant congenital cataract. Slit-lamp examination showed nuclear cataracts.
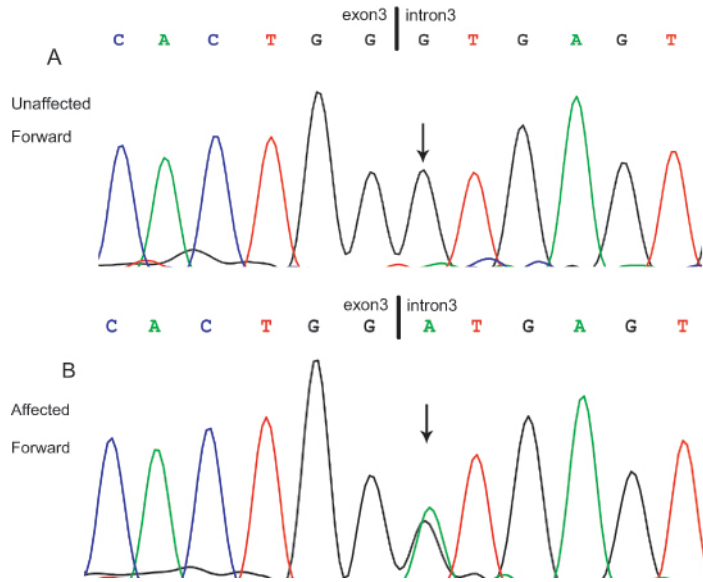
Figure 3. Identification of a novel mutation (c.606+1 G>A) in the major intrinsic protein (*MIP*) gene in a Chinese family with autosomal dominant congenital cataract. **A**: DNA sequencing results of an unaffected family member (upper panel) and an affected family member (lower panel). Results showed a heterozygous mutation (c.606+1 G>A) at the donor of splice site of intron 3 of the *MIP* (black arrow) gene. The black vertical line shows the normal exon 3-intron 3 donor splice site.

mice, and knockout mice have been shown to trigger bilateral cataracts [11,15,16]. This information further supports the conclusion that this protein plays a vital role in normal lens development.

To date, 11 mutations in *MIP* have been reported to associate with autosomal dominant cataracts, including eight missense mutations (c.702G>A [17], c.559C>T [7], c.530A>G [18], c.401A>G [19], c.319G>A [20], c.97C>T [21], c.413C>G [19]), and c.494 G >A [22], one acceptor splice-site mutation (c.607–1G>A) [6], one deletion that causes a frameshift at 638delG(c.638delG) [23], and one initiation codon mutation

(c.2T>C) [24]. These mutations can cause different phenotypes and protein dysfunction. In this way, they affect the permeability and trafficking of water.

Splice donor sites and splice acceptor sites are highly conserved during evolution. Mutations at these splice sites have been found to be associated with various genetic disorders. Splice-site mutations usually lead to abnormal pre-mRNA splicing, which results in exon skipping, activation of cryptic splice sites, creation of pseudo-exons within introns, and intron retention [25]. The mRNA levels of the *MIP* gene in blood specimens are extremely low [4]. Thus, a
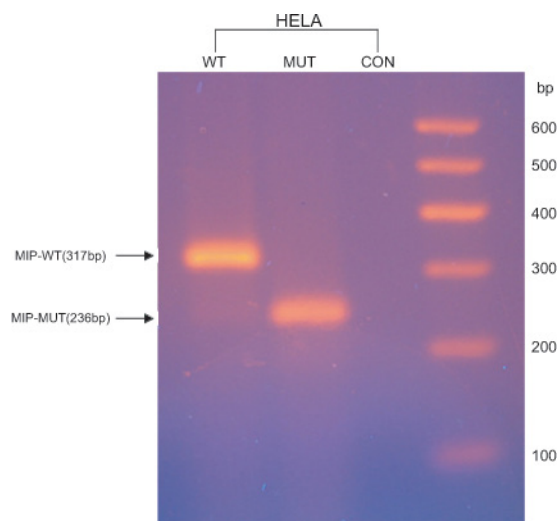

Figure 4. Reverse transcription–polymerase chain reaction results of minigene constructs. Reverse transcription–polymerase chain reaction (RT–PCR) products obtained from HeLa cells were transfected with pcDNA3.1-MIP-WT, pcDNA3.1-MIP-MUT, and pcDNA3.1(+)independently. RT–PCR products were separated on a 2% agarose gel. RT–PCR of total RNA obtained from cells transfected with pcDNA3.1-MIP-WT showed a 317 bp band, and RT–PCR of total RNA obtained from cells

transfected with pcDNA3.1-MIP-MUT showed a 236 bp band. No bands were detected when RT–PCR was performed with total RNA from pcDNA3.1 (+) transfected cells.
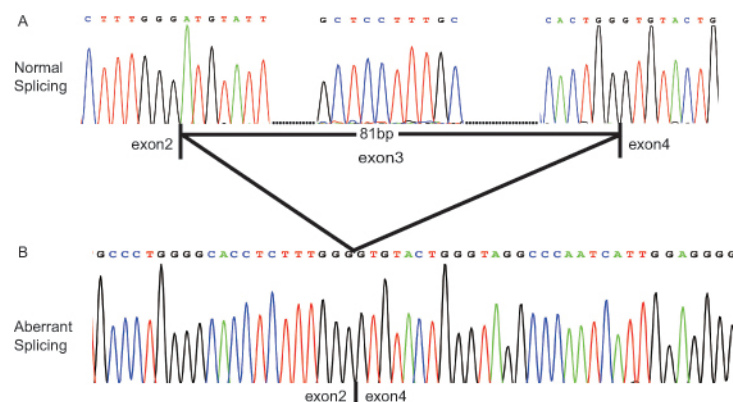
Figure 5. DNA sequencing of wild-type and mutant reverse transcription–polymerase chain reaction products. **A**: Normal splicing shows the mRNA contains exon 3. **B**: Aberrant splicing shows that exon 3 was deleted.

minigene splicing experiment was used to test the effects of the splice-site mutations. It was then demonstrated that the donor splice-site mutation (c.606+1G>A) caused exon 3 to be deleted from the MIP mRNA, which led to the deletion of 27 amino acids from the MIP protein. The deletion of 27 amino acids may further affect the function of the MIP protein.

In summary, this is the first report to show that the donor splice-site mutation (c.606+1 G>A) in the *MIP* gene causes autosomal dominant congenital cataract. A minigene splicing assay was used to confirm that the mutation resulted in the deletion of exon 3 from the MIP mRNA. Omitting exon 3 leads to the deletion of 27 amino acids from the MIP protein, which then may affect the function of the MIP protein. This study expands the spectrum of mutations that cause congenital cataracts.

REFERENCES

1. Hejtmancik JF. Congenital cataracts and their molecular genetics. Semin Cell Dev Biol 2008; 19:134-49. [PMID: 18035564].

2. Santana A, Waiswo M. The genetic and molecular basis of congenital cataract. Arq Bras Oftalmol 2011; 74:136-42. [PMID: 21779674].

3. Hejtmancik JF, Smaoui N. Molecular genetics of cataract. Dev Ophthalmol 2003; 37:67-82. [PMID: 12876830].

4. Jin C, Jiang J, Wang W, Yao K. Identification of a MIP mutation that activates a cryptic acceptor splice site in the 3′ untranslated region. Mol Vis 2010; 16:2253-8. [PMID: 21139677].

5. Yang Z, Su D, Li Q, Yang F, Ma Z, Zhu S, Ma X. A novel T→G splice site mutation of CRYBA1/A3 associated with autosomal dominant nuclear cataracts in a Chinese family. Mol Vis 2012; 18:1283-8. [PMID: 22665976].

6. Jiang J, Jin C, Wang W, Tang X, Shentu X, Wu R, Wang Y, Xia K, Yao K. Identification of a novel splice-site mutation in MIP in a Chinese congenital cataract family. Mol Vis 2009; 15:38-44. [PMID: 19137077].

7. Wang KJ, Li SS, Yun B, Ma WX, Jiang TG, Zhu SQ. A novel mutation in MIP associated with congenital nuclear cataract in a Chinese family. Mol Vis 2011; 17:70-7. [PMID: 21245956].

8. Agre P. Membrane water transport and aquaporins: looking back. Biol Cell 2005; 97:355-6. [PMID: 15901244].

9. Preston GM, Carroll TP, Guggino WB, Agre P. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science 1992; 256:385-7. [PMID: 1373524].

10. Gonen T, Walz T. The structure of aquaporins. Q Rev Biophys 2006; 39:361-96. [PMID: 17156589].

11. Shiels A, Bassnett S, Varadaraj K, Mathias R, Al-Ghoul K, Kuszak J, Donoviel D, Lilleberg S, Friedrich G, Zambrowicz B. Optical dysfunction of the crystalline lens in aquaporin-0-deficient mice. Physiol Genomics 2001; 7:179-86. [PMID: 11773604].

12. Chepelinsky AB. The ocular lens fiber membrane specific protein MIP/Aquaporin 0. J Exp Zool A Comp Exp Biol 2003; 300:41-6. .

13. Nemeth-Cahalan KL, Hall JE. pH and calcium regulate the water permeability of aquaporin 0. J Biol Chem 2000; 275:6777-82. [PMID: 10702234].

14. Mathias RT, Kistler J, Donaldson P. The lens circulation. J Membr Biol 2007; 216:1-16. [PMID: 17568975].

15. Chepelinsky AB. Structural function of MIP/aquaporin 0 in the eye lens; genetic defects lead to congenital inherited cataracts. Handbook Exp Pharmacol 2009; 190:265-97. .

16. Shiels A, Bassnett S. Mutations in the founder of the MIP gene family underlie cataract development in the mouse. Nat Genet 1996; 12:212-5. [PMID: 8563764].

17. Lin H, Hejtmancik JF, Qi Y. A substitution of arginine to lysine at the COOH-terminus of MIP caused a different binocular phenotype in a congenital cataract family. Mol Vis 2007; 13:1822-7. [PMID: 17960133].

18. Yang G, Zhang G, Wu Q, Zhao J. A novel mutation in the MIP gene is associated with autosomal dominant congenital nuclear cataract in a Chinese family. Mol Vis 2011; 17:1320-3. [PMID: 21647270].

19. Berry V, Francis P, Kaushal S, Moore A, Bhattacharya S. Missense mutations in MIP underlie autosomal dominant 'polymorphic' and lamellar cataracts linked to 12q. Nat Genet 2000; 25:15-7. [PMID: 10802646].

20. Wang W, Jiang J, Zhu Y, Li J, Jin C, Shentu X, Yao K. A novel mutation in the major intrinsic protein (MIP) associated with autosomal dominant congenital cataracts in a Chinese family. Mol Vis 2010; 16:534-9. [PMID: 20361015].

21. Gu F, Zhai H, Li D, Zhao L, Li C, Huang S, Ma X. A novel mutation in major intrinsic protein of the lens gene (MIP) underlies autosomal dominant cataract in a Chinese family. Mol Vis 2007; 13:1651-6. [PMID: 17893667].

22. Senthil Kumar G, Kyle JW, Minogue PJ, Dinesh Kumar K, Vasantha K, Berthoud VM, Beyer EC, Santhiya ST. An MIP/AQP0 mutation with impaired trafficking and function underlies an autosomal dominant congenital lamellar cataract. Exp Eye Res 2013; 110:136-41. [PMID: 23116563].

23. Geyer DD, Spence MA, Johannes M, Flodman P, Clancy KP, Berry R, Sparkes RS, Jonsen MD, Isenberg SJ, Bateman JB. Novel single-base deletional mutation in major intrinsic protein (MIP) in autosomal dominant cataract. Am J Ophthalmol 2006; 141:761-3. [PMID: 16564824].

24. Xiao X, Li W, Wang P, Li L, Li S, Jia X, Sun W, Guo X, Zhang Q. Cerulean cataract mapped to 12q13 and associated with a novel initiation codon mutation in MIP. Mol Vis 2011; 17:2049-55. [PMID: 21850180].

25. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet 1992; 90:41-54. [PMID: 1427786].