Novel mutations in GJA3 associated with autosomal dominant congenital cataract in the Indian population

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Purpose: Connexin 46 (Cx46) is crucial in the maintenance of lens homeostasis and it is known to be expressed mainly in the terminally differentiated lens fiber cells. The present study aimed to identify the spectrum of mutations in Connexin 46 in the Indian population.

Methods: PCR based Single Stranded Conformational Polymorphism (SSCP) analysis was used to screen sixty probands with nonsyndromic congenital cataract for mutations in the Cx46 gene (GJA3), followed by direct sequencing of samples that showed an electrophoretic shift. Mutation predicted to affect the coding sequence were subsequently analyzed in the entire pedigree.

Results: Two novel missense mutations were identified in Cx46. The mutation in Family 1 was characterized as R76G with a total cataract phenotype. A V28M missense mutation was identified in family 2, the cataract phenotype varied in its severity and the age of onset. The mutation was also identified in 2 unaffected individuals of the family and the intrafamilial variation of the disease suggests the possibility of a modifier gene(s) or the effects of environmental factors being involved. The mutation was identified in all the affected members in the family and found to be absent in 400 ethnically matched control chromosomes analyzed.

Conclusions: We conclude that connexin 46 mutations might account for as much as 3.3% of the hereditary congenital cataract in the Indian population.

Cataracts as lens opacities have been recognized as a group of well-known diseases for centuries. Congenital cataracts are one of the most common major eye abnormalities, and often lead to blindness in infants. Since the visual system undergoes a critical stage of development during infancy and early childhood, the management of congenital cataract is more complicated than its age related counterpart. It can occur as a primary disorder or secondarily in association with multisystem disorders such as Down’s syndrome, galactosemia, Wilson disease, and myotonic dystrophy [1].

Genetic causes are implicated in half of all congenital and developmental cataracts [2]. Within this group highly penetrant Autosomal Dominant forms of Congenital Cataract (ADCC) are the most common and are both phenotypically and genetically heterogeneous [3]. Autosomal recessive [4] and X-linked forms are also known to exist [5].

To date more than 30 loci have been reported to be associated with various forms of congenital cataract. Among them, thirteen loci have been associated with isolated congenital cataracts, with specific gene mutations. These include genes for structural proteins like crystallins (CRYAA, CRYAB, CRYBA1, CRYBB1, CRYBB2, CRYGC, and CRYGD) [6-11], the cytoskeletal proteins (BFSP2) [12], lens membrane proteins (GJA3, GJA8, MIP, and LIM2) [4,13-15], and transcriptional factors (HSF4) [16].

The lens, being an avascular system, cell-to-cell communication via gap junctions is essential for the maintenance of lens homeostasis. An essential role for gap junctional communication in the lens has been suggested by the findings that mutations in either the human connexin 46 (Cx46) or connexin 50 (Cx50) proteins cause cataracts [13,14] and that lens opacities develop in mice with targeted deletions of these connexins [17,18]. This study aimed to identify the spectrum of mutation in Cx46 of patients with congenital cataract in the Indian population.

METHODS

Clinical documentation: Probands with a positive family history of nonsyndromic, congenital, or developmental cataract up to the age of 30 years were included in the study. Probands with a history suggestive of an intrauterine infection such as rubella, complicated cataract, unilateral cataract, and traumatic cataract were excluded from the study. Informed consent was obtained to draw blood from identified cases attending the Pediatric Ophthalmology Clinic of Aravind Eye Hospital (AEH), Madurai, India. Detailed ocular, medical, and family histories were obtained. Ophthalmic examination included tests of visual function such as Snellen visual acuity, corrected visual acuity, slit-lamp, and fundus examination with dilated pupil. Photographs of significant findings were taken depending upon the co-operation of the patient.
A total of sixty pedigrees including 32 families with autosomal dominant inheritance, 18 autosomal recessive inheritance, and 10 small families of uncertain inheritance were recruited in this study. The control subjects for the study were recruited from the general ophthalmology clinic of AEH matching the ethnic background of the probands. The study was performed in accordance to the Tenets of Declaration of Helsinki and with the approval of the Institutional Review Board and Ethics Committee of the Hospital.

**DNA preparation and single strand conformation polymorphism (SSCP):** Venous blood (5 ml) was collected for genomic DNA extraction following the salt precipitation method as described by Miller et al. [19].

Cx46 is encoded by **GJA3** located in chromosome 13q11 that consists of a single exon coding for 435 amino acids. PCR was carried out to amplify the exonic region of **GJA3** for all the probands using the primers previously published [13,20].

The amplified products were diluted with an equal volume of loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 98 °C for 5 min followed by snap cooling on ice. Denatured amplicons were loaded onto 12% polyacrylamide gel and electrophoresed at 700 V for 10-12 h at room temperature and the gels were silver stained according to the modified protocol of Bassam et al. [21].

**DNA sequence analysis:** PCR products that demonstrated a mobility shift in SSCP gels were re-amplified using the same set of primers and column purified using the QIA quick gel extraction kit (QIAGEN, Hilden, Germany) and sequenced using dye terminator chemistry on an Applied Biosystem (ABI, Rotkerz, Switzerland) model 377 automated sequencer.

**Restriction digestion:** The transversion of C>G at position 226 (Genbank NM_021954) leads to a R76G mutation that results in the loss of a **BseGI** restriction site. Codon 29-103 was amplified using the primer pair 5'-CTG TTC A TC TTC CGC A TC TTG G-3' and 5'-TCC A TG CGC ACG A TG TGC AGC A-3' that resulted in a 224 bp product. Restriction digestion with **BseGI** was carried out at 55 °C for 3 h according to the manufacturer’s instructions (MBI Fermentas, Vilnius, Lithuania). Digestion products were analyzed on a 2.5% agarose gel.

Restriction digestion was carried out to confirm the presence of V28M mutation in the family that resulted in gain of an **NlaIII** recognition site. The region harboring the mutation was amplified using the primer pair 5'-TGC GGA CCC GGC ACT CAG C-3' and 5'-TCC ATG CGC ACG ATG TGC AGC A-3' which resulted in a 383 bp fragment from all the available family members and digested using **NlaIII** at 37 °C in NEB buffer 4 for 2 h (New England Biolabs, Beverly, MA), the products were electrophoresed on a 1.5% agarose gel.

### TABLE 1. SNPs in human **GJA3** in subjects of Indian origin

| cDNA    | Amino acid | Frequency |  |  |
|---------|------------|-----------|  |  |
| c.84 G>A| V28V       | 1/60      | 0/100     |  |
| c.252 C>T| F84F    | 0/60      | 1/100     |  |
| c.375 A>G| P125P    | 1/60      | 1/100     |  |
| c.393 G>A| S131S    | 1/60      | 1/100     |  |

Variants identified in the connexin 46 gene which were found nonsegregating with the cataract phenotype.

Figure 1. Analysis of family 1. **A:** Clinical picture of the proband showing total cataract phenotype. **B:** The pedigree of the proband. The shaded circle (female) and shaded boxes (males) represent the affected members and the unshaded boxes and circles denote the unaffected members in the family. An arrow indicates the proband. **C:** Restriction endonuclease analysis of the family. Wild type individuals display a 142 bp and 82 bp band while affected individuals, due to the loss of a **BseGI** site in one of the alleles, display 224 bp, 142 bp, and 82 bp cleavage products. A 100 bp standard molecular weight ladder is in lane M, undigested DNA is in lane UD, and a restriction analysis of an unrelated healthy control is in lane C.
RESULTS

Analysis of the probands of sixty unrelated family members revealed two unique heterozygous mutations. In addition four new nonsegregating single nucleotide polymorphisms were identified in the population screened (Table 1).

Family 1: This two generation family had three affected members with total cataract phenotype (Figure 1A,B). The cataract was most likely caused by a point mutation (226C>G) that led to the replacement of Arg by Gly at a highly conserved codon 76 of GJA3 (Figure 2). The co-segregation of the mutation with the disease phenotype was confirmed by the loss of a BsaI restriction site (Figure 1C). The residue is predicted to be in the first extra cellular loop as derived from the GenBank sequence (Q9Y6H8) and to be in the boundary of first extra cellular loop and second trans membrane domain as described by Burdon et al. [22].

Family 2: A variable cataract phenotype was detected in this family. The Proband (V:7) and her mother (IV:4) suffered from total cataract that was apparent within the first month of birth, where as the other members (III:6, IV:1, V:1, V:2) were diagnosed to have cataract after 20 years of age. Individual V:2 (aged 24) was identified to have posterior cortical opacities with anterior capsular cataract and individual V:1 (aged 26) was identified to have peripheral cortical opacities (Figure 3A,B). Sequencing of GJA3 from the proband showed a G>A transition at position 82, resulting in a V28M substitution. This G>A change resulted in the gain of a novel NlaIII recognition site (CATG) in one of the alleles. Restriction digestion was carried by amplifying the respective fragment harboring the mutation from all the available family members for determining the co-segregation of the mutant allele with the diseased phenotype. This analysis revealed two other unaffected members in the family (V:3, V:4) with the same change, which was not observed in 400 control chromosomes analyzed.

DISCUSSION

Gap junctions are plasma membrane spatial micro domains constructed of assemblies of channel proteins called connexins in vertebrates, which facilitate direct intercellular communication pathways. These channels allow rapid exchange of ions, secondary messengers, and metabolites up to 1 kDa in size between cells [23]. In the eye three members of the connexin family are known to be expressed. Connexin 43 (Cx43) encoded by GJA1 is expressed mainly in lens epithelial cells where as Connexin 46 encoded by GJA3 and Connexin 50 encoded by GJA8 are expressed in terminally differentiated lens fibers [24].

In an animal model study the targeted replacement of connexin 50 with the connexin 46 coding region in mice demonstrated that Cx50 is required for cell growth whereas Cx46 provided nonspecific restoration of intercellular communication [25]. Seven of the previously reported Cx46 mutations in humans have been reported to cause ADCC with nuclear or zonular pulverulent cataract (Table 2).

In all the connexins, the greatest sequence conservation is observed in the extracellular loops and transmembrane domains. In family 1 the 226C>G base change resulted in a R76G mutation. It is not clear if the R76 residue is located on the first extracellular loop or the second transmembrane domain. If the nonconservative substitution of a basic polar amino acid Arg by an uncharged polar amino acid Gly at the highly conserved codon 76 lies on the first extra cellular loop (E1) it might alter the charge on the surface of the extra cellular loop thereby affecting the connexon docking [26]. The E1 loop has also been shown to be important for the determination of transjunctional voltage required for the closure of gap junction pores and the change in charge might affect the normal functioning of the connexin [27]. If the mutation lies on the transmembrane domain it might affect the correct transport of protein into the plasma membrane [28]. In the Australian popul...
Figure 3. Analysis of Family 2. A: Photograph of the anterior eye with lens image of individual V:2, showing anterior capsular cataract with posterior cortical opacities. B: Retroillumination of the lens of individual V:1, showing peripheral cortical opacity. C: Pedigree of family 2. The shaded circle (female) and shaded boxes (males) represent the affected members and the unshaded boxes and circles denote the unaffected members in the family. The slash mark through the box/circle indicates deceased. An arrow indicates the proband. D: Restriction fragment length analysis showing a gain of an NlaIII site in the mutant that results in a 341 bp, 222 bp, 119 bp, 40 bp, and 2 bp fragments. The wild type displays 341 bp, 40 bp, and a 2 bp fragment. The 40 bp and 2 bp bands are not visible on the agarose gel.
lation the R76H mutation has been identified to be incompletely penetrant and has been reported to cause a pulverulent cataract phenotype [21], whereas the R76G mutation reported in this study led to opacification of the total lens.

The V28M missense mutation is the second mutation to be reported in the first transmembrane domain of connexin 46 and is highly conserved among all the connexins at position 28 (Figure 2). While the precise contribution of each domain to the formation of the pore of gap junction and therefore channel permeability, remains controversial, the first transmembrane domain has been strongly implicated in this process [29,30].

There is an intrafamilial variation in the cataract phenotype and its severity in family 2. Individuals V:3 (aged 15) and V:4 (aged 12) carrying the mutation were thoroughly examined and are clearly still not affected. There is a possibility that these individuals may develop cataract at a later stage as their other siblings (V:1 and V:2) and their mother (IV:1) did. It is clear from the pedigree that there is delayed onset of cataract in individuals (III:6, V:1, V:2), when compared with individuals IV:4 and V:7 who developed cataract immediately after birth (Figure 3C,D). Because these individuals have inherited the same mutation in the GJA3, other factors must be responsible for this variation in phenotype. Possible explanations include a modifier gene or genes and undetermined environmental influences. The results of this family are consistent with that of an animal study wherein targeted deletion of Gja3 in mice developed nuclear cataract shortly after birth. A large variance in the severity of cataract was observed in Gja3 null sibs of 129SvJ genetic background when backcrossed with C57Bl/6J background, suggesting the phenotype was influenced by genetic background [31].

To conclude, although the number of unrelated individuals in our study was not large enough to provide accurate estimates, we have found connexin 46 mutations in 2 of 60 families studied, which suggested that mutations in these genes might account for as much as 3.3% of inherited congenital cataracts in the Indian population. This renders GJA3 to be one of the most common non-crystallin genes to be associated with congenital cataract and confirms its role in the pathogenesis of autosomal dominant congenital cataract.

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<table>
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Connexin 46 gene mutations identified in the present study and other previous studies which were associated with autosomal dominant congenital cataract.
(Aravind Medical Research Foundation, Madurai, India), Dr. Jochen Graw (GSF, National Research Center, Neuherberg, Germany), and Dr. J Fielding Hejtmancik (National Eye Institute, NIH, Bethesda, MD) for helpful discussions. Thanks are also due to Jeya krishnan, Muthulakshmi, and Gomathy for their technical assistance. This study was supported by a grant through the Aravind Eye Hospital.

REFERENCES

of congenital cataract hereditary includes autosomal dominant, auto-somal recessive, or X-linked, among which autosomal dominant is the most common [2]. Autosomal dominant congenital cataracts (AD-CC) as congenital cataracts are genetically heterogeneous, which is known that the highly variable morphologies of cataracts within some families. A 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. Five potential pathogenic mutations were confirmed in the five probands associated with congenital cataract: the heterozygous mutation c.154 T > C (p.F52 L) in GJA8 in Family 1, c.1152_1153insG (p.S385Fs*83) in GJA3 in Family 2, and c. 1804 G > C (p.G602R) in BFSP1 in Family 3, c.1532C > T (p.T511 M) in EPHA2 in Family 4 and mutation c. 356G > A (p. R119H) in HSF4 in Family 5. The five mutations were novel and were first identified as associated with congenital cataract. A We reported five novel mutations associated with the autosomal dominance cataract in five Chinese families respectively: c.154 T > C in GJA8, c.1152_1153insG in GJA3, c.1804G > C in BFSP1, c.1532C > T in EPHA2 and c.356G > A in HSF4. Autosomal dominant congenital cataract (ADCC) is a clinically and genetically heterogeneous ocular disease in children that results in serious visual impairments or even blindness. Targeted exome sequencing (TES) is an efficient method used for genetic diagnoses of inherited diseases. Among these, more than 20 causative genes for these loci have been associated with ADCC, and the number of the identified genes is increasing[7, 8]. Half of the identified mutations in the ADCC family are crystallin genes, including CRYAA, CRYAB, CRYBA1/A3, CRYBA4, CRYBB1, CRYBB2, CRYBB3, CRYGC, CRYGD and CRYGS, another one-quarter are gap junction. Using this approach, a novel heterozygous mutation in GJA3 was revealed in this family. @article{Devi2005NovelMI, title={Novel mutations in GJA3 associated with autosomal dominant congenital cataract in the Indian population.}, author={R. Devi and C. Reena and P. Vijayalakshmi}, journal={Molecular vision}, year={2005}, volume={11}, pages={846-52 } }. R. Devi, C. Reena, P. Vijayalakshmi. Published 2005. Biology, Medicine. Molecular vision. PURPOSE Connexin 46 (Cx46) is crucial in the maintenance of lens homeostasis and it is known to be expressed mainly in the terminally differentiated lens fiber cells. Purpose: To identify the genetic defect in an autosomal dominant congenital cataract family, having 15 members in three generations, affected with bilateral cataract that gave the appearance of “full moon” with Y-sutural opacities. Methods: A detailed family history and clinical data were recorded. A Mutation screening in GJA8 identified a novel G>C transversion at nucleotide position c.235. This nucleotide change resulted in the substitution of highly conserved valine by leucine at codon 79 (V79L). This nucleotide substitution was neither seen in any unaffected member of the family nor in 180 unrelated control subjects (360 chromosomes) from same ethnic background tested by sequence analysis of GJA8.