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Novel mutations in *GJA8* associated with autosomal dominant congenital cataract and microcornea

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Purpose: The purpose of this study was to estimate the importance of mutations in the connexin50 gene (*GJA8*) as a cause of congenital or developmental cataracts in the Indian population and to identify novel mutations in *GJA8* that cause cataract in this population.
Methods: The coding region of *GJA8* was analyzed for mutation by single strand conformational polymorphism in 60 probands affected with congenital or developmental cataract of which 11 probands' corneal diameter measured less than 11.00 mm. Direct sequencing was performed for samples that displayed an abnormal electrophoresis pattern. The segregation of the change with the diseased phenotype was analyzed in the entire pedigree by restriction fragment length polymorphism (RFLP) analysis.
Results: Molecular analysis of *GJA8* revealed two novel missense mutations V44E and R198Q, in the population screened. The mutations cosegregated with the diseased phenotype in an autosomal dominant manner and were absent in 400 normal control chromosomes analyzed. *GJA8* mutations were seen in two of the 60 unrelated probands with cataracts. Affected individuals in both of whose families also had microcornea and variable myopia.
Conclusions: This is the first report of mutations in *GJA8* to be associated with autosomal dominant cataract and microcornea. Mutations in *GJA8* cause 3.3% of congenital cataracts in the population of India.

Cataract or opacification of all or part of the lens in the eye is a major ocular disease that renders millions of people blind throughout the world. Congenital cataract is the most common cause of treatable childhood blindness. Worldwide, 20 million children under the age of 16 suffer from cataract and 1.4 million of them are blind [1]. Inherited cataract accounts for at least 50% of all congenital cataracts [2]. Hereditary cataracts are most commonly inherited in an autosomal dominant manner and are phenotypically and genotypically heterogeneous, showing considerable inter- and intrafamilial variability [3]. Autosomal recessive and X-linked forms are also known to exist [4,5].

The lens is an avascular structure that has an anterior layer of cuboidal epithelial cells that terminally differentiate at the equatorial zone to form lens fiber cells [6]. Fully differentiated lens fibers lose their intracellular organelles and accumulate high concentrations of soluble proteins known as crystallins. Initially, these cells are arranged as concentric layers extending from the anterior to the posterior surface of the lens, but as the lens develops, the lens fiber cells extend from the capsule to the lens sutures. Lens fiber cells lack nuclei, mitochondria, and ribosomes, so that they must be physiologically maintained to prevent precipitation of crystallin and cataract formation. Since the lens lacks any vasculature, this is achieved by highly sophisticated protein architecture of intercellular junctions, which allow the metabolically active epithelium to maintain the precise intercellular communication and transport between the lens periphery and its interior [7].

Connexin genes encode intercellular channels that in diffusion of signaling molecules regulate growth and development. These channels allow rapid exchange of ions and metabolites up to about 1 kDa in size [8]. Connexins are a multigene family consisting of >20 members, three of which are expressed in the lens [9]. The lens epithelial cells show a predominant expression of connexin 43 (Cx43) [10]. During differentiation into fibers, Cx43 expression is down regulated and replaced by Cx46 and Cx50 [11,12]. Targeted ablation of Cx46 and Cx50 in mice caused cataract and genetics studies in humans revealed that mutations in either of these genes (*GJA3* and *GJA8*, respectively) lead to the development of distinct cataract phenotypes [13-16]. This studys aims were to analyze GJA8 in Indian patients affected with congenital/childhood cataract.

METHODS

Patients: Probands with a positive family history of congenital cataract or developmental cataract were ascertained through the Paediatric Ophthalmology Clinic, Aravind Eye Hospital (AEH), Madurai, India. Probands with a history suggestive of intrauterine infection, such as rubella, complicated cataract, unilateral cataract, and traumatic cataract were excluded from the study. Ethics approval for the study was obtained from the Institutional Review Board and the study was performed in accordance to the tenets of Declaration of Helsinki. Blood samples were collected from the proband and their available

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family members after obtaining informed consent. The control subjects who matched the ethnic background of the proband were recruited from the general ophthalmology clinic of the AEH. Ophthalmologic examination included the best corrected visual acuity (Snellen's), slit lamp biomicroscopy, and fundus examination. Corneal diameter and axial length were measured when indicated.

Mutation analysis: DNA was isolated from peripheral blood lymphocytes using protocols described by Miller et al. [17]. Connexin 50 encoded by *GJA8* located on chromosome 1q21.1 is comprised of two exons, the coding sequence being encompassed in its entirely by the second exon (NM_005267). Polymerase chain reaction (PCR) was used to amplify the coding exon using oligonucleotide primer pairs and PCR conditions as previously described in the literature [18]. One volume of PCR product was mixed with 1 volume of formamide dye and denatured at 98 °C for 4 min prior to loading on an 8% nondenaturing gel. Electrophoresis was carried out at 700 V for 12-15 h. Direct sequencing was performed for product that displayed a difference in electrophoresis pattern from that of the controls. Primers flanking codon 1-108 and 169-318 were used to amplify the fragment harboring the mutation for RFLP analysis to test the identified sequence variants in the family members of the proband for segregation of the disease. The segregating sequence variants with the diseased phenotype were analyzed for the presence in 400 normal control chromosomes.

RESULTS

Sixty probands of unrelated families with inherited congenital or developmental cataract, including 11 probands with cataract and microcornea, were enrolled in this study. The probands of each of these families were analyzed for *GJA8* mutations, which revealed significant changes in two probands. In both proband families all affected members were diagnosed with congenital cataract associated with microcornea. The clinical phenotypes are summarized in Table 1.

In family 1, six members (two affected and four unaffected) participated in the study. The affected members had congenital cataract and microcornea. The proband had total lens opacification. She is a glaucoma suspect postoperatively (intraocular pressure is high but has normal fundus). The axial length of individual III:1 (R-25.65, L-25.95) suggested a mild degree of myopia. Mutation analysis revealed c.131T>A transversion that led to the replacement of evolutionarily conserved valine at position 44 by glutamic acid (V44E; Figure 1A,B,E). The mutation resulted in the gain of a *Taq*I restriction site. RFLP analysis confirmed the presence of a heterozygous T>A transversion in the affected members of the pedigree (Figure 1D).

Family 2 was comprised of three generations, with three affected members (Figure 2C). The proband (III:2) was diagnosed with bilateral developmental cataract. The axial length was 25 mm in both eyes, suggestive of mild myopia. The type of cataract was posterior subcapsular. Medical records of individuals I:1 and II:1 show that they underwent cataract extraction at 27 and 10 years of age, respectively. The cataract phenotype was not available. Individual II:1 is diagnosed with high myopia; the axial length was 29.5 mm in the right eye and 24.5 mm in the left eye. The decreased axial length in the left eye is a complication of an old retinal detachment, which led to the impairment of vision in the eye. The mutation in this family was identified as a c.593G>A transition that resulted in a missense mutation where a highly conserved arginine was replaced by glutamine (R198Q; Figure 2A,B,E). The loss of a *Msp*I restriction site in the mutant allele was used to confirm the cosegregation of the mutation with the disease phenotype (Figure 2D).

In both families, individuals with the mutation had cataract with microcornea variably associated with myopia. The mutation segregated with the disease phenotype in the respective families and was not present in 400 normal controls analyzed. The GenBank sequence (P48165) revealed that residue V44 was in the first transmembrane domain and R198 on the second extra cellular loop.

DISCUSSION

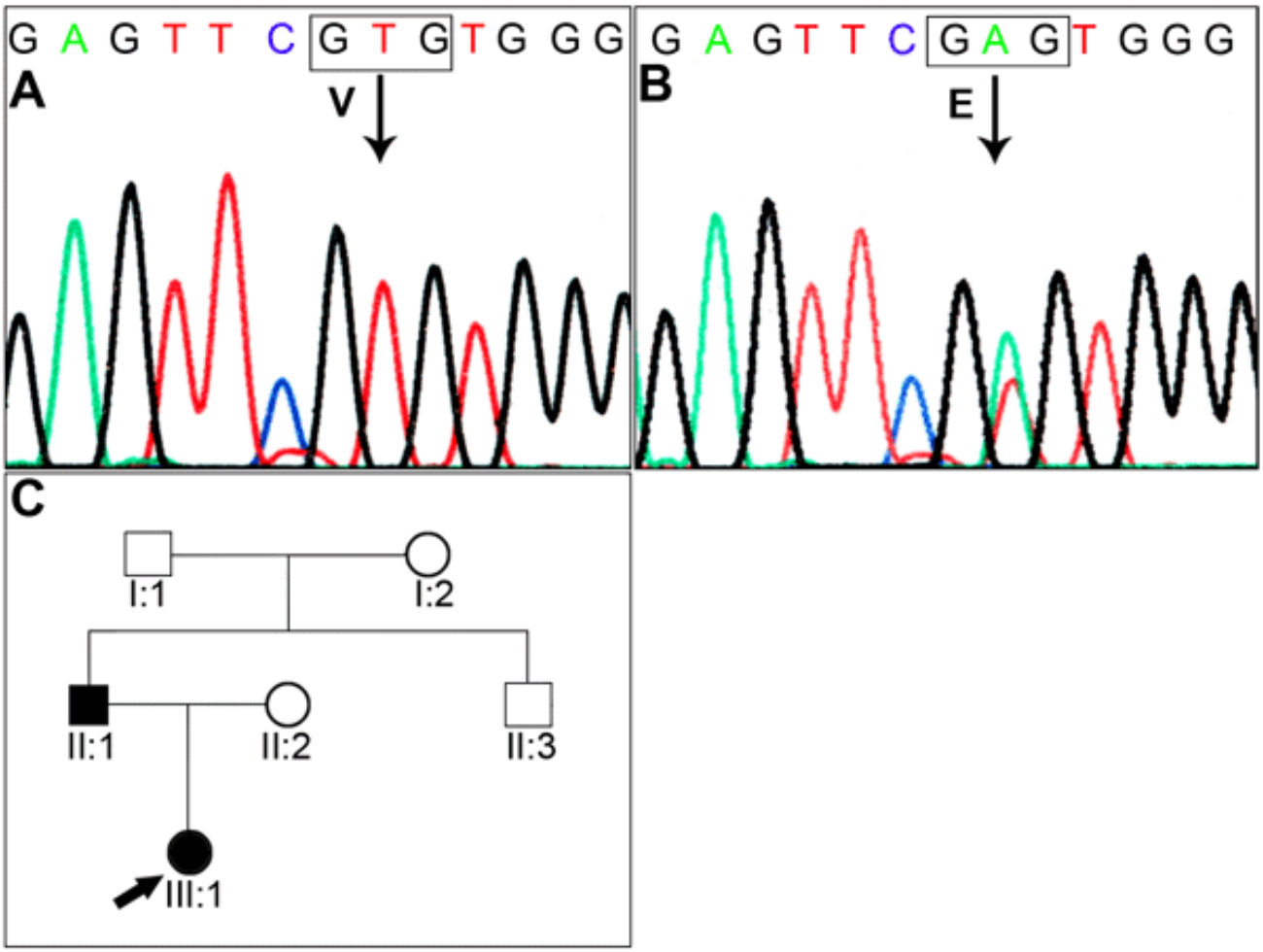
We report two novel missense mutations in connexin 50 that is shown to be responsible for congenital cataract and microcornea, while previous reports of mutations in Cx50 (summarized in Table 2) are associated with isolated congenital cataract.

Table 1. Clinical findings of individuals with the *GJA8* mutation

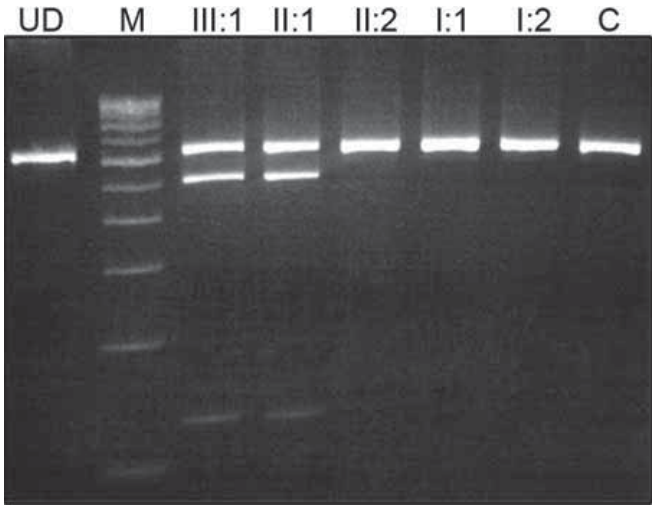
				Snellen best corrected vision		Corneal diameter (mm)		Axial length (mm)	
Family	Individual	Age (years)	Age at cataract surgery	Right	Left	Right	Left	Right	Left
1	II:1	40	3 years	6/36	6/36	10.5	10.5	25.65	25.95
	III:1	6	3 months	6/24	6/24	10.0	10.0	19.50	21.03
2	I:1	57	22 years	6/60	6/60	10.0	10.0	21.05	20.98
	II:1	32	10 years	6/60	HM	10.0	10.0	29.50	24.50
	III:1	7	4 years	2/60	2/60	10.5	10.5	25.21	25.06

The column "Age" refers to the age at which corneal diameter (microcornea is defined as corneal diameter <11 mm) and axial length was measured. "HM" refers hand motions.

Microcornea is defined by the horizontal corneal diameter measuring less than 11.00 mm. The association of cataract with microcornea in the absence of other ocular anomalies has been observed in rare pedigrees [19,20]. Microcornea and cataract occurs in association with other ocular manifestations such as microphthalmia, myopia, iris coloboma, sclerocornea, and Peter's anomaly [21,22].



D:



E:

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CXA8_HUMAN    11   LEEVNEHSTVIGRVWLTVLFIFRILILGTAAEFVWGDEQS   50
CXA8_MOUSE    11   LEEVNEHSTVIGRVWLTVLFIFRILILGTAAEFVWGDEQS   50
CXA8_SHEEP    11   LEEVNEHSTVIGRVWLTVLFIFRILILGTAAEFVWGDEQS   50
CXA8_CHICK    11   LEQVNEQSTVIGRVWLTVLFIFRILILGTAAELVWGDEQS   50
CXA1_HUMAN    11   LDKVQAYSTAGGKVWLSVLFIFRILLLGTAVESAWGDEQS   50
CXA3_HUMAN    11   LENAQEHSTVIGKVWLTVLFIFRILVLGAAAEDVWGDEQS   50
CXA4_HUMAN    11   LDQVQEHSTVVGKIWLTVLFIFRILILGLAGESVWGDEQS   50
CXA5_HUMAN    11   LEEVHKHSTVVGKVWLTVLFIFRMLVLGTAAESSWGDEQA   50
CXA10_HUMAN   11   LEEVHIHSTMIGKIWLTILFIFRMLVLGVAAEDVWNDEQS   50
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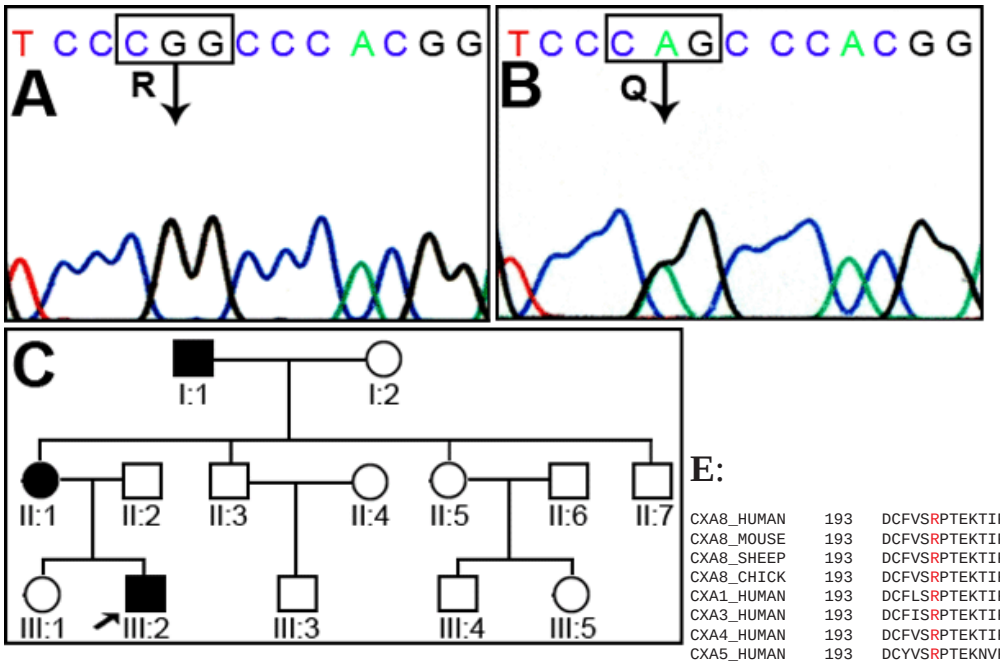
Figure 1. Analysis of family 1. **A**: The genomic sequence of the wild type showing valine at codon 44. **B**: The mutant allele showing a heterozygous T>A transversion that replaces Val by Glu. **C**: Pedigree of family 1. **D**: PCR-RFLP analysis showing a gain of a *Taq*I site that cosegregates with the affected individuals. The unaffected individuals display a 648 bp band while the affected individuals display a 648 bp, 519 bp, and 129 bp bands. In the image, M indicates the 100 bp DNA ladder, C indicates control, and UD indicates undigested PCR product. **E**: A multiple alignment of amino acid sequence of GJA8 with different species and five other connexins from human. The amino acid marked in red indicates a neutral amino acid (valine, alanine, or serine) at codon 44.



E:

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CXA8_HUMAN    193   DCFVSRPTEKTIFILFMLSVASVSLFLNVMELGHLGLKGIR   233
CXA8_MOUSE    193   DCFVSRPTEKTIFILFMLSVAFVSLFLNIMEMSHLGMKGIR   233
CXA8_SHEEP    193   DCFVSRPTEKTIFILFMLSVASVSLFLNILEMSHLGLKKIR   233
CXA8_CHICK    193   DCFVSRPTEKTIFIMFMLVVAAVSLFLNLVEISHLILKRIR   233
CXA1_HUMAN    193   DCFLSRPTEKTIFIIFMLVVSLVSLALNIIELFYVFFKGVK   233
CXA3_HUMAN    193   DCFISRPTEKTIFIIFMLAVACASLLLNMLEIYHLGWKKLK   233
CXA4_HUMAN    193   DCFVSRPTEKTIFIIFMLVVGLISLVLNLLELVHLLCRCLS   233
CXA5_HUMAN    193   DCYVSRPTEKNVFIVFMLAVAALSLLLSLAELYHLGWKKIR   233
CXA10_HUMAN   193   DCFVSRPTEKTIFLLFMQSIATISLFLNILEIFHLGFKKIF   233
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D:

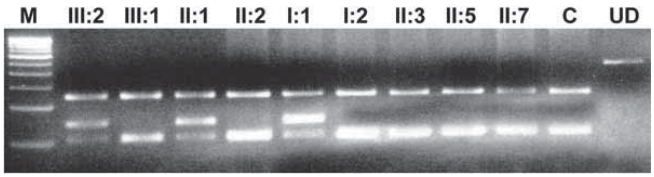

Figure 2. Analysis of family 2. **A**: The genomic sequence of the wild type pedigree showing an arginine at codon 198. **B**: The mutant allele showing a heterozygous T>A transition that substitutes Glu for Arg. **C**: Pedigree of family 2. **D**: PCR-RFLP analysis showing the loss of an *Msp*I site in the mutant allele that results in 243 bp, 144 bp, 27 bp, 21 bp, and 16 bp bands while the normal allele shows 243 bp, 105 bp, 39 bp, 27 bp, 21 bp, and 16 bp bands. The latter 39 bp, 27 bp, 21 bp, and 16 bp bands are not visible on the gel. In the image, M indicates the 100 bp DNA ladder, C indicates control, and UD indicates undigested PCR product. **E**: A multiple sequence alignment of Cx50 with other connexin proteins. The amino acid marked in red indicates the position of R198, where arginine is highly conserved among connexins.

Table 2. Summary of mutations in human *GJA8*

Base change	Mutation	Location	Population	Reference
c.68 G>C	R23T	Cytoplasmic N-terminal	Iranian	[16]
c.131 T>A	V44E	First transmembrane domain	Indian	This study
c.142 G>A	E48K	First extracellular loop	Pakistani	[37]
c.191 T>G	V64G	First extracellular loop	Chinese	[38]
c.262 C>T	P88S	Second transmembrane domain	British	[18]
c.593 G>A	R198Q	Second extracellular loop	Indian	This study
c.741 T>G	I247M	Cytoplasmic C-terminal	Russian	[39]

Shown are connexin 50 gene mutations that have been identified in this and other studies.

Mutations identified in transcription factor gene such as *PAX6* have led to cataract, microcornea, and anterior segment dygenesis, while mutations in *MAF* resulted in pulverulent cataract variably associated with microcornea, and iris coloboma [23,24]. A missense mutation in *CRYAA* and a homozygous nonsense mutation in *CRYBB2* have been reported to cause cataract, microcornea and microphthalmous [25,26]. To date, the X253R mutation in *CRYBB1* is the only report to be associated with cataract and microcornea in the absence of other ocular or systemic anomalies [27], and cataract associated with myopia has been described as a result of a *bfsp2* mutation [28].

Connexin 50 is a gap junction protein that forms an array of intercellular channels that are established by docking of hemichannels, one from each of two closely apposed cells. A hemichannel is comprised of a hexamer of connexin subunits. All connexins are composed of four transmembrane domains linked by two extracellular loops that have highly conserved amino acid sequence identity, and a single highly variable intracellular loop and an intracytoplasmic NH_2- and COOH-terminal [29,30].

In family 1, the nonconservative substitution of the negatively charged polar glutamic acid for an uncharged nonpolar valine at position 44 is predicted to lie on the first transmembrane domain (Figure 1). The transmembrane domains of the connexins are proposed to participate in the oligomerization into connexon hemichannels and are also essential for the correct transport of the protein into the plasma membrane. It has been identified that pore lining residues lie in the first transmembrane domain and are essential for the formation of the pore and therefore channel permeability [31]. In family 2 the c.593G>A change results in a R198Q missense mutation. The replacement of the highly conserved basic polar ariginine by the uncharged nonpolar glutamine lies on the second extracellular loop (Figure 2E). The extracellular loops are crucial for the docking of the two hemichannels to generate a gap junction unit, and it has been demonstrated that primary sequence of second extracellular loop plays a role in determining the compatibility of heterotypic channel formation among different lens connexins [32]. This is the first report of missense change in the first transmembrane domain and the second extracellular loop, which highlights the functional importance of this region of the protein.

Cataractogenesis has been observed in both Cx46 and Cx50 knockout mice. In contrast to the Cx46 knockout, the target ablation of Cx50 mice resulted in reduced growth of the lens and eye [13,14]. Targeted replacement of Cx50 with Cx46 prevented cataract but did not restore normal ocular growth [33]. The reduction in the total ocular growth is possibly due to the reduction in lens growth, as it has also been proposed from the chick studies that normal lens development is essential for the normal growth of the eye [34]. Naturally occurring missense mutation G22R in Lop10 mice led to cataract and mice homozygous for the mutation developed microphthalmia with dense cataract [35]. A similar effect was observed in the No2 mouse mutation (D47A) where heterozygous mice developed milder cataract and mice homozygous for the mutation exhibited a reduction in the total ocular mass by approximately 30% compared with wild-type [36]. It would be interesting to study the effect of V44E and R186Q mutation on the ocular phenotype of mice.

This is the first report of mutations in *GJA8* to be associated with autosomal dominant cataract and microcornea. Though the etiology of microcornea in these families is not clear, mutations disrupting the protein structure severely and thereby interfering with the lens development can affect the anterior segment development.

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