Detection of Mutation c.139G>A (D47N) in GJA8 and c.2036C>T in FYCO1 Gene in an Extended Family with Inheritance of Autosomal Dominant Zonular Cataract without Pulverisation Pulverulent opacities by Exome Sequencing

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Abstract

**Purpose:** To identify the gene causing bilateral autosomal dominant zonular congenital cataract (ADZCC) without pulverulent opacities in an extended Muslim family by exome sequencing and subsequent analysis.

**Methods:** An extended family of 37 members (14 affected and 23 unaffected) belonging to different nuclear families were screened for causative gene. Proband and her unaffected son were screened for causative variant by exome sequencing followed by Sanger sequencing of the proband’s entire nuclear family. Remaining members were further screened for variants detected, by PCR-RFLP and Tetra ARMS PCR.

**Results:** Review of exome sequencing data of the proband and her unaffected son for 40 known genes causing congenital non-syndromic cataracts revealed two variants viz., c.139G>A (**p.Asp47Asn; D47N**) in **GJA8** gene and c.2036C>T in **FYCO1** gene to be potentially pathogenic. Further, validation of these two variants in the entire family showed co-segregation of c.139G>A variant in **GJA8** with ADZCC without pulverulent opacities. Variation c.2036C>T in **FYCO1** was not associated with disease in the family.

**Conclusion:** The mutation c.139G>A in **GJA8** gene detected in the present study was also previously described in Caucasian and Chinese families but with different phenotypes i.e. nuclear and nuclear pulverulent cataracts. Thus, the mutation c.139G>A in **GJA8** appears to exhibit marked interfamilial phenotypic variability.
INTRODUCTION

Cataract, the opacification of lens, results from alteration in cellular architecture of lens, lens proteins or both. It is a major ocular disease that causes blindness in millions of people throughout the world. Opacification of the ocular lens is caused by a wide range of etiological factors including exogenous factors like infections, chemicals, radiations etc. All these factors have their effect right from intrauterine life to senescence. Further, lens opacity shows considerable interfamilial and intrafamilial phenotypic variation in the expression of different types of cataracts that are reported worldwide (Scott et al, 1994; Vanha, 1998; Amaya et al, 2003). Depending on the morphological, clinical and pathological profiles, the lens opacities acquire an independent identity like cortical, nuclear, zonular, lamellar, crystalline, total and other types. These different types may originate either congenitally or during infantile, juvenile, presenile or senile stages of life. Congenital cataracts exhibit clinically and genetically heterogenous lens disorders and account for approximately 10% of the worldwide cases of childhood blindness (Francis et al, 2000). They occur either as syndromic or non-syndromic form with different modes of inheritance of which autosomal dominant mode of transmission is most commonly reported (Amaya et al, 2003).

To date, about 40 genes have been reported to be associated with non-syndromic congenital cataracts, among which twenty nine genes are known to cause autosomal dominant pattern of inheritance (Cat-map, Table-1). Formation of these cataracts is caused by mutations in different families of genes which include lens related crystalline genes (CRYAA, CRYAB, CRYBB1, CRYBB2, CRYBB3,CRYBA1, CRYBA4, CRYGB, CRYGC, CRYGD, and CRYGS), connexin genes (GJA3, GJA8), membrane protein genes (AGK, CHMP4B, EPHA2, MIP, and LIM2), cytoskeleton-related genes (BFSP1 and BFSP2), transcription factor genes (HSF4, MAF, PITX3) and others (CCA5, CCPSO, CTAA1, CTAA2, CTP1, CTRCT29, CTRCT35, FYCO1, GCNT2, LONP1, LSS, NHS, UNC45B, VIM, WDR87, WFS1 and WNT3). Some of the genes viz., BFSP2, CRYAB, CRYAA, CRYBA1, CRYBB1, CRYBB3, EPHA2, GJA8, HSF4 and PITX3 are reported to cause both dominant as well as recessively inherited congenital cataracts. Among the different genes involved, nearly 50% of the cataract types are caused by mutations in crystalline genes followed by mutations in the genes encoding for connexins (Sheils et al, 2010).
Connexins are gap junction proteins that play an important role in intercellular communication and maintenance of lens homeostasis. They are composed of four transmembrane domains linked by two extracellular loops that have highly conserved amino acid sequence identity, and a highly variable intracellular loop and an intracytoplasmic NH2- and COOH- terminal (Hertzberg et al, 1988; Bennett et al, 1991). The genes GJA3 and GJA8 encoding Connexin 46 and Connexin 50 respectively are known to account for 20% of non-syndromic cataracts with different types of phenotypes reported worldwide (He and Li, 2000). To-date, more than 43 mutations have been reported in the coding region of GJA8 gene of which 19 are known to be associated with non-syndromic autosomal dominant pattern of inheritance (Cat-map). GJA8 (NM_005267) gene comprising two exons is located on chromosome 1q21. Only exon2 in the gene codes for a 50KD protein. Mutations that are reported in GJA8 gene leading to the formation of non-syndromic autosomal dominant congenital cataracts are enlisted in Table-2. So far, 7 mutations causing congenital cataracts (5-autosomal dominant & 2-autosomal recessive) are reported in GJA8 gene from India. Of these, only 1 mutation was associated with non-syndromic autosomal dominant cataract where the phenotype was full moon with Y sutural opacities (Vanita et al, 2006). The remaining mutations were associated with other phenotypes such as micro cornea with mild myopia (Devi and Vijayalaxmi, 2006; Vanita et al, 2008) and nystagmus (Ponnam et al, 2007; Kumar et al, 2011).

In the present study, an extended Muslim family settled long back in India from Hyderabad, India with bilateral autosomal dominant zonular cataracts with no traces of pulverisation pulverulent opacities was analysed to detect the pathogenic mutation causing the condition by exome sequencing of the affected proband and her unaffected son. Two pathogenic variants namely c.139G>A (rs121434643) in GJA8 gene and c.2036C>T (rs3796375) in FCOI gene were detected by exome sequencing followed by Sanger sequencing in the nuclear family. PCR-RFLP and Tetra ARMS PCR methods were designed to screen the two variants among rest of the family members to detect their co-segregation with the inherited dominant zonular cataract.

**MATERIALS AND METHODS**

*Clinical evaluation*
An extended four generation Muslim family with inheritance of bilateral congenital zonular cataracts with no traces of pulverisation, pulverulent opacities registered at Medivision Eye Care Center, Hyderabad, India was investigated to identify the causative gene segregating in the family. The couple in the first generation were not alive but the male member of the family was informed to be affected with cataract during his childhood. Among their progeny and grand and great grand children, 37 members in the family, comprising 14 affected and 23 unaffected individuals were available for our study (Fig-1). 37 individuals belonged to different nuclear families of which only one family was complete with 5 members including parents and three children. The mother i.e. the proband in the nuclear family and her two daughters were affected with congenital zonular cataract whereas her husband and son were normal. In the entire family there was no history of consanguinity. Clinical and ophthalmological examinations including screening for visual acuity, slit lamp and fundus examination of all the 14 affected members did not reveal history of any other ocular abnormalities apart from the zonular cataract. The peripheral embryonic and peripheral infantile nuclei were found to be transparent and in foetal tissue the opaque zone was detected. So the origin of zonular cataract among the affected members was considered as foetal. The cataract phenotypes were documented by slit lamp photography (Fig-2). Written consent was obtained from all the members who participated in the study and the study was approved by the institutional ethical committee and adhered to the guidelines of the Declaration of Helsinki.

Collection of Blood Samples
Peripheral venous blood samples were collected in EDTA vacutainers from all the members who co-operated to participate in the study. Genomic DNA was extracted using conventional chloroform method as described by Dahm (Dahm, 2008). The DNA was quantitated using Nanodrop (Thermoscientific) by recording O.D at 260nm.

Whole exome sequencing
Whole exome sequencing was used to identify the pathogenic mutation causing autosomal dominant zonular cataract in the extended family as it is a powerful and cost effective tool for identifying the variants of various genes. We selected the female proband and her unaffected son from the nuclear family within the extended family for exome sequencing. The criteria for selecting only these two samples was (1) The entire family with five members was
available for the study (2) The family consisted of both affected and unaffected offspring and (3) Only one of the two parents was affected.

Exome sequencing was performed by a commercial service, Sandor Life Sciences Pvt Ltd. Exome capture was carried out using an Illumina TruSeq Exome Enrichment Kit (62 M) that covered about 97.2% consensus coding sequence. Exome-enriched DNA fragments were sequenced by an Illumina HiSeq2000 with the average sequencing depth being 125-fold. Over 99% base call accuracy was up to Q20, which means that the probability of an incorrect base call is 0.01.

After the low quality reads were filtered, the clean data was aligned to the consensus sequence (UCSC hg19) to detect variants by SAMtools. Additional bioinformatics analysis of all the variants was obtained from dbSNP (http://www.ncbi.nlm.nih.gov/), OMIM (http://www.omim.org/), 1000 Genome (http://browser.1000genomes.org/index.html), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/).

**Variant Analysis**

The results of exome sequencing of the proband and her unaffected son were compared for variants in 40 known causative genes reported in Cat-map database (http://cat-map.wustl.edu/; Table-1) for non-syndromic congenital cataracts. Then we excluded the variants which we didn’t consider as pathogenic using the following criteria: 1) Minor allele frequency (MAF) ≥ 0.01 from 1000 Human Genome Project database; 2) Located in non-coding region without affecting splicing site; 3) Synonymous variants without affecting splicing site and 4) Only one single heterozygous variation detected in recessive genes. Only variants having possibly damaging effect on the protein were considered as potentially pathogenic and were summarized for validation.

**Sanger Sequencing**

Sanger sequencing was done to confirm the potential pathogenic variants detected by exome sequencing in the nuclear family through commercial source (Eurofins Genomics India Pvt Ltd). The variants that were confirmed by Sanger sequencing in the five samples belonging to nuclear family were further screened in the rest of the family members and 120 unrelated normal healthy controls by Polymerase chain reaction Restriction fragment length polymorphism (PCR-RFLP) and Tetra ARMS PCR. Primers to amplify the regions with each
variant for Sanger sequencing, PCR RFLP and Tetra ARMS PCR were designed using Batch Primer 3 software (http://probes.pw.usda.gov/batchprimer3/; Table 23). RFLP protocol was designed using NEB Cutter (http://nc2.neb.com/NEBcutter2/).

**PCR RFLP**

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) was used for screening c.139G>A variation in exon2 of GJA8 gene. PCR amplification was carried out in a total reaction volume of 10µl containing 1µl PCR buffer, 200mMeach dNTP, 0.25U Taq polymerase, and 2.5 pmol each of forward and reverse primers. The PCR conditions to amplify the marker included initial denaturation at 94°C for 5 m, followed by 30 cycles of denaturation at 94°C for 35 s, annealing at 54°C for 30 s, extension at 72°C for 35 s and a final extension at 72°C for 7m. Restriction digestion was carried out by incubating 5 ml of the PCR product with 2U of FokI restriction enzyme overnight. Then genotyping was done by electrophoresis on polyacrylamide gels. Homozygous wild types (GG) were determined by the presence of three fragments of 199bp, 56bp and 33bp and heterozygotes (GA) by four fragments of 199bp, 89bp, 56bp and 33bp. None of the samples showed homozygosity for the mutant allele.

**Tetra ARMS PCR**

Tetra ARMS PCR was used to detect the presence of c.2036C>T variation in exon 8 of FYCO1 gene. The tetra ARMS PCR method involves the use of two primer pairs to amplify two different alleles in one PCR reaction. PCR reaction was carried out in a total reaction volume of 10µl containing 1µl PCR buffer, 200mMeach dNTP, 0.25U Taq polymerase, and primers at a ratio of 3:1 (inner to outer). PCR conditions included initial denaturation at 94°C for 5 m, followed by 40 cycles of denaturation at 94°C for 1 m, annealing at 65°C for 1m, extension at 72°C for 45 s and a final extension at 72°C for 7m. Genotyping was done by agarose gel electrophoresis and the alleles were interpreted based on the size of the fragments generated which was 208bp for allele T and 287bp for allele C.

**RESULTS**

Whole exome sequencing was used to identify the causative mutation segregating among the members of the extended family affected with bilateral autosomal dominant zonular cataract without pulverisation, pulverulent opacities and other ophthalmic symptoms by testing two
samples one from affected mother (proband) and another from her unaffected son belonging to the nuclear family.

The results of whole exome sequencing revealed the presence of 49 variants in the proband and 65 variants in her unaffected son which are related to 40 known causative genes reviewed for non-syndromic congenital cataracts. After excluding all the non-pathogenic variants and comparing the variants detected in the proband and her unaffected son, two variants, c.139G>A (p.Asp47Asn; D47N)) in GJA8 gene and the other c.2036C>T (p.Ala679Val) variant in FYCO1 gene were chosen for follow up since they were considered as potentially pathogenic, found to have possible damaging effect and therefore were considered potentially pathogenic. Presence of these two variants in all the five members of the nuclear family was confirmed by following Sanger sequencing.

**Analysis of c.139G>A in GJA8 gene**

Screening of c.139G>A variation in the nuclear family by Sanger sequencing (Fig-3) revealed the proband and her two affected daughters to be heterozygous for c.139G>A variant in GJA8 gene while her unaffected son and husband were found to be homozygous normal. Screening of rest of the members of the extended family revealed co-segregation of c.139G>A mutation with the inherited autosomal dominant zonular congenital cataract without pulverisationpulverulent opacities. To confirm further, the causative nature of c.139G>A, we genotyped 115 unrelated normal healthy individuals from the same population and 222 cases with age related cataract. None of the controls and age related cataract cases showed the variation detected in GJA8 gene supporting the causative nature of c.139G>A to the development of congenital zonular cataract in the family.

**Analysis of c.2036C>T in FYCO1 gene**

Considering the variation c.2036C>T (rs3796375) in FYCO1 gene, Sanger sequence (Fig-4) of all the 5 members of the nuclear family were found to be homozygous mutants. Analysis of the remaining family members by Tetra ARMS PCR revealed that inheritance of FYCO1 variation was independent from the inheritance of congenital zonular cataract among the members of the extended family. Further, analysis of the variation in 222 age related cataract cases did not show any susceptibility of this variation to the development of cataract formation. It is reported to be a common variant in various populations across the world. Further screening of the variation in 115 unrelated normal healthy individuals revealed that...
the variation was polymorphic in nature with minor allele frequency >0.01. Further, analysis of the variation in 222 age-related cataract cases did not show any susceptibility of this variation to the development of cataract formation.

DISCUSSION

Congenital cataracts are one of the most common eye disorders leading to blindness in children worldwide. Of the mutations known to be associated with congenital cataracts, approximately one quarter of them are located in the connexin genes. Both connexin 46 (Cx46) and connexin 50 (Cx50), encoded by GJA3 and GJA8 respectively, are reported to be associated with autosomal dominant congenital cataracts (Jiang, 2010). Studies have shown that these transport membrane proteins are vital for the proper embryological development of the lens. They are essential for maintaining lens transparency, and GJA8 is required for proper fiber cell proliferation and control of lens transparency (Gong et al, 2010). Moreover, Cx50 is required for pH mediated gating of gap junction channels in differentiating fibers and mutations in the gene could alter the electrical properties of gap junction channels. To date, more than 43 mutations have been identified in different domains of GJA8 gene that contribute to inherited congenital cataracts with clear cut phenotypic variability.

The mutation c.139G>A (p.Asp47Asn; D47N) detected in the present study is located in the interface between the first transmembrane domain and the first extracellular loop and replaces the negatively charged aspartic acid at position 47 by the highly conserved polar, uncharged asparagine. The amino acid position 47 in connexin 50 is a mutational hotspot comprising various variants viz., D47Y, D47H and D47N. Previously this mutation was identified in a Caucasian family with nuclear pulverulent cataract (Arora et al, 2008). So far majority of mutations reported in GJA8 gene were found to be associated with either nuclear pulverulent or zonular pulverulent cataracts.
The present study differs from these reports as it is the first report indicating the co-segregation of mutation c.139G>A in GJA8 gene causing replacement of asparagine by aspartic acid at position 47 with autosomal dominant zonular cataract without pulverisation, pulverulent opacities and with foetal origin. This shows that mutations in Cx50 exhibit significant interfamilial phenotypic variability.

Considering the mutation c.2036C>T (p.Ala679Val) in FYCO1 gene, it was inherited independently from the disease in the present family. FYCO1 encodes a binding protein involved in autophagosome trafficking and was found to be associated with congenital cataracts (Chen et al, 2011). Recessive mutations in the gene are rare but are responsible for 10% of recessive type of cataract in Pakistan (Chen et al, 2011). Chen et al, (2011) reported 9 different pathogenic mutations in FYCO1 gene in 12 Pakistani families with bilateral nuclear congenital cataract and 1 Arab Israeli family with posterior lenticonus affecting the intracellular transport of phagocytic vesicles. These mutations were shown to affect the intracellular transport of phagocytic vesicles. Khan et al, (2015) identified 2 mutations (c.2505del and c.449T>C in FYCO1 gene) in two families with bilateral posterior capsular abnormalities. In addition, one splice variant was reported by Gillespie et al, (2014) in their study using next generation sequencing to enhance the diagnosis of congenital cataracts.

It is interesting to note that the variation c.2036C>T in FYCO1 gene detected in the present study was not associated with either congenital or age related cataracts though it was found to be possibly pathogenic to have damaging effect on the protein. This variant lies 170bp away from the c.2206C>T variant found to be associated with congenital cataracts in a Pakistani family reported by Khan et al, (Chen et al, 2011). Among other diseases, the variation in FYCO1 gene detected by us was found to be associated with microscopic colitis in a study by Garner et al, (2014). This shows that phenotypic variability may exist in the expression of FYCO1 gene variants and hence the mutations in the gene may not always cause cataract formation. The members in the family studied did not report any other significant disease condition.

In conclusion, we report a pathogenic heterozygous c.139G>A mutation in GJA8 (connexin 50) in an extended family with bilateral autosomal dominant zonular cataract without pulverisation, pulverulent opacities that showed marked phenotypic difference from previously reported cases viz., nuclear and nuclear pulverulent cataracts with the same substitution. There is possibility of occurrence of digenic or modifier genes in the genome as...
observed in case of non-syndromic hearing impairment (Pallares Ruiz et al, 2002; Bronya et al, 2006) influencing the variability in the expression of lens opacification associated with same gene mutations. Though c.139G>A mutation in GJA8 gene was a major cause of development of autosomal dominant zonular cataract in the present study, the mutation detected in FYCO1 gene was also evaluated to verify if there was any interaction—co-segregation of between the two genes GJA8 and FYCO1, causing opacification of the lens in the family studied.

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References


Figure legends

**Figure-1**: Pedigree showing the segregation of c.139G>A in GJA8 and c.2036C>T in FYCO1 gene in an extended family with autosomal dominant zonular cataract without pulverisation. Arrow denotes the affected proband in the nuclear family with 5 members indicated by asterix. Black and open symbols denote affected and unaffected individuals respectively. H denotes heterozygosity, N homozygous normal and M homozygous mutant genotypes for both GJA8 and FYCO1 gene variants. Number in the male and female symbol denotes no. of sibs.

**Figure-2**: Illustrates the appearance of zonular cataracts without pulverulent opacities among affected members of the family. A) Diffused illumination that shows opacification in central part of the lens B) Slit lamp photograph of lens with clear central embryonic nucleus and clear peripheral lens. Cataract development is seen only in the fetal nucleus.
Figure-3: Chromatogram showing partial genomic sequence of GJA8 gene. a) Sequence of an unaffected member from nuclear family and b) Sequence of an affected member from the nuclear family. Arrow indicates the mutation c.139G>A in GJA8 gene.

Figure-4: Chromatogram showing partial genomic sequence of a member of nuclear family with c.2036 C>T substitution in FYCO1 gene. Arrow indicates the mutation.