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A Novel Gap Junction Alpha 8 (GJA8) Mutation Associated with a Congenital Cataract Patient in Pakistan

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AZ did the experimental work. SM helped in blood sampling. AM wrote the article. MSM helped in bioinformatics analysis. RS submitted sequence to NCBI. SI supervised the work.

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Congenital cataracts, GJA8, Mutation screening, PCR-SSCP.

ABSTRACT

Cataracts are principal cause of visual impairments among people, although ocular surgery can reestablish vision in such patients but genetic researches have validated that, mutations in *GJA8* are coherent source of lens opaqueness and inappropriate growth of fiber cells. In the present study, a novel G to C substitution (1104G>C) (pE368Q) was screened by PCR-SSCP in exon 2 of GJA8 and this tansversion altered exceedingly conserved glutamic acid to glutamine at site which was involved in coding of ASF1 like histone chaperone. Further presumption based on structural and functional analysis of mutated protein was anticipated by bioinformatics tools, which manifest mild changes in overall charge but altered post translational modifications in a way which might have a deleterious effect on ion channels anatomy and on the whole, pave ways to the genesis of cataract.

INTRODUCTION

Congenital cataract is one of the pre-eminent sources of visual impairment among children and reported for one tenth instance of visual loses in them (Lambert and Drack, 1996; Wang *et al*., 2011). It affects 0.6-6 out of 10,000 infants in developed countries and 5-15 per 15,000 in developing countries (Francis *et al*., 2000; Reddy *et al*., 2004; Holmes *et al*., 2003; Apple *et al*., 2000). It can occur in solitary or in compound state which affected miscellaneous tissues (Hu *et al*., 2010). Not all the congenital cataract cases are genetic, only 50% are which have multiple geneses; they may be autosomal dominant, autosomal recessive and X-linked (Vanita *et al*., 2009).

At present congenital cataract is associated with mutation in more than 18 known genes that include; *FTL, CRYGC, CRYBB2, CRYBA1, EPHA2, CRYAB, CHMP4B. GJA8. GJA3, CRYGD, DMPK, MIP, BFSP2, PITX3, CTDP1, SIL1, RAB3GAP1, RAB3GAP2, RAB 18, GJA1, RECQL4, DHCR7,CRYBB3, NDP* and *NHS*, located at divergent chromosomes (Xu and Traboulsi, 2014; Chen *et al*., 2015). One half of the mutations are associated with genes that code for crytallins, while a quarter with genes that encoded connexin and remaining to other genes that encrypted chromatin modifying protein-4B, beaded heat shock transcription factor-4, filament structural proteins-2, lens intrinsic membrane protein 2, avian musculoaponeurotic fibrosarcoma, paired-like homeodomain transcription factor-3, Eph-receptor type-A2 and major intrinsic protein or aquaporin-0 (Hejtmancik, 2008; Huang and He, 2010).

Cataract exhibits a variety of morphologies that comprises anterior polar, pyramidal, anterior lenticonus, cortical lamellar, fetal nuclear, posterior polar, posterior lentiglobus, posterior subcapsular, persistent fetal vasculature (PFV) and traumatic disruption of lens. It is important to identify the proper morphology that disclosed its etiology and ultimate possible prognosis and cure (Wilson, 2015). Lens cells in eye, accomplished intracellular communication via an immense network of gap junctions formed by the structural proteins belongs to connexin family, to permit the trafficking of ions and small solutes of size $\leq$ 1 kDa (Girelli *et al*., 2001). Connexin 50 (GJA8, Cx50) and Connexin 46 (GJA3, Cx46) together build up the gap junctions. At present nearly 34 different mutations have been specified in GJA8 gene and most of the reported mutations were missense (Sarkar *et al*., 2014).

Here, we detect a novel mutation in Cx50 (GJA8) gene in one of the congenital cataract patient, in Pakistan, by the use of PCR-SSCP. This may help to comprehend the role of this mutation in the prognosis of disease.

MATERIALS AND METHODS

Sample collection

After receiving the ethical approval from hospital authorities and patients, 27 clinically diagnosed congenital

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cataract cases with age ranges from 1-8 years old, were selected for the study from different parts of Punjab province, at Children Hospital, Lahore Pakistan. These congenital cataract cases had no other ocular or systemic abnormalities. The complete history of the patients was taken by knowing the status of affected patients. A total of 27 age-matched normal individuals without any ocular or systemic abnormalities were certain as control.

DNA Isolation and PCR amplification

DNA isolation of controls and patients samples was performed using the standard protocol (Miller *et al*., 1988). DNA spectrometry was used for quantitative analysis of isolated genomic DNA and agarose gel electrophoresis (with 1% agarose) was used for qualitative analysis. The GJA8 exon 2 was amplified in congenital cataract patients and controls. PCR amplification was performed for all the primer sets (Table I) in a 25 µl volume mixture containing 20 mM of each primer, 100 ng of genomic DNA, 1 unit of Taq polymerase, 10 mM of dNTPs and 10 X PCR buffer. PCR condition was set with an initial denaturation for 10 min at 94 °C, followed by 35 cycles of denaturation for 45 seconds at 94 °C, annealing for 45 seconds at 58 °C and extension for 45 seconds at 72 °C with a final extension for 10 min at 72 °C. Amplified PCR product was observed on 1.5% agarose gel.

Table I.- Sequence of the Oligonucleotide primers (Kumar *et al*., 2011).

Gene	Sequences	Tm (ºC)
GJA8-(1)-F	5′-TATGGGCGACTGGAGTTTCCT-3′	57.8
GJA8-(1)-R	5′-CTCCATGCGGACGTAGTGCAC-3′	61.7
GJA8-(2)-F	5′-CTCTGGGTGCTGCAGATCATC-3′	59.8
GJA8-(2)-R	5′-CACAGAGGCCACAGACAACAT-3′	57.8
GJA8-(3)-F	5′-CACTACTTCCTGTACGGGTTC-3′	57.8
GJA8-(3)-R	5′-CTCTTGGTAGCCCCGGGACAA-3′	61.7
GJA8-(4)-F	5′-GTCTCCTCCATCCAGAAAGCC-3′	59.8
GJA8-(4)-R	5′-TCATACGGTTAGATCGTCTGA-3′	53.9

SSCP and sequence analysis

Single stranded polyacrylamide gel analysis with 8% polyacrylamide was done to identify novel mutations in GJA8 gene. Amplified products were purified by using Fermentas GeneJET Gel Extraction Kit (#K0691, #K0692). Purified products were sequenced by First Base Laboratories (Sdn Bhd No. 7-1 to 7-3, Jalan SP 2/7, Taman Serdang Perdana, Seksyen 2, 43300 Seri Kembangan, Selangor, Malaysia). Sequencing results were analyzed by using BLAST and Clustal Omega.

Bioinformatics analysis

I-TASSER, was used for the prediction of secondary structure of wild type protein along with its prophesized 3-D structure, by using 10 most appropriate threading templates, which have been nominated on the basis of their Z-score. PROVE and ERRAT were used for the authentication of anticipated structure. Ramachandran plot of wild type protein was also plotted for the estimation of energetically stable amino acids. SWISS MODEL was used for the assessment of superimposed structure of wild type and mutated proteins for the scrutiny of possible structural variations. Stability of wild-type, as well as the mutated model, is calculated by FoldX software.

Functional analysis

Functional analysis of modified protein was done by using online tools HOPE and MutPred.

RESULTS

Clinical assessment

The cataract cases included in this study had no consanguineous marriage or any other family history. Total 27 congenital cataract cases with age group ranges from 1-8 years old, were included in this study. Most of them were infants. In the following study, 17 cases were male and 10 were female. The time of onset of disease was the age when it was first observed or detected by parents and doctors at the Ophthalmology section of Children Hospital, Lahore.

Mutational analysis

Genomic DNA was isolated according to standard procedure. The standard PCR, with all primer sets was performed for affected and control samples. The amplified products were run on 8% SSCP-PAGE for analyses of their banding pattern. SSCP-PAGE results showed that polymorphism exists in the amplified region of exon 2 of GJA8 in one of the patient (Fig. 1). PCR product of patient sample which showed mobility was sequenced for further analysis.

Sequence analysis

Sequence of normal individual matched perfectly with the reported sequence of GJA8 exon 2 which was retrieved from NCBI (GenBank NG_016242.1) (Fig. 2).

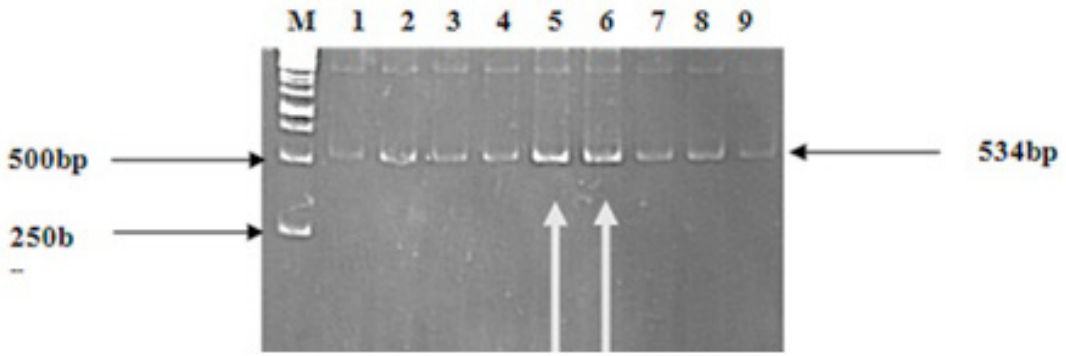

Fig. 1. SSCP banding pattern of PCR products of GJA8 exon 2 of patient samples affected 10 to affected 17, with primer set 4. M, 1Kb DNA ladder; Lane 1, A10; Lane 2, A11; Lane 3, A12; Lane 4, A13; Lane 5, A14; Lane 6, A14; Lane 7, A15; Lane 8, A16; Lane 9, A17. White arrowheads indicate an extra band in Lane 5 and 6.

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Normal      ----------------CCCCTGGGGGAGATTCCTGAGAAATCCCTCCACTCCATTGCTG     43
Reported    AGAGGCCTGTAGAGCAGCCCCTGGGGGAGATTCCTGAGAAATCCCTCCACTCCATTGCTG     780
                            *******************************************

Normal      TCTCCTCCATCCAGAAAGCCAAGGGCTATCAGCTCCTAGAAGAAGAGAAAATCGTTTCCC     103
Reported    TCTCCTCCATCCAGAAAGCCAAGGGCTATCAGCTCCTAGAAGAAGAGAAAATCGTTTCCC     840
            ************************************************************

Normal      ACTATTTCCCCTTGACCGAGGTTGGGATGGTGGAGACCAGCCCACTGCCTGCCAAGCCTT     163
Reported    ACTATTTCCCCTTGACCGAGGTTGGGATGGTGGAGACCAGCCCACTGCCTGCCAAGCCTT     900
            ************************************************************

Normal      TCAATCAGTTCGAGGAGAAGATCAGCACAGGACCCCTGGGGGACTTGTCCCGGGGCTACC     223
Reported    TCAATCAGTTCGAGGAGAAGATCAGCACAGGACCCCTGGGGGACTTGTCCCGGGGCTACC     960
            ************************************************************

Normal      AAGAGACACTGCCTTCCTACGCTCAGGTGGGGGCACAAGAAGTGGAGGGCGAGGGGCCGC     283
Reported    AAGAGACACTGCCTTCCTACGCTCAGGTGGGGGCACAAGAAGTGGAGGGCGAGGGGCCGC     1020
            ************************************************************

Normal      CTGCAGAGGAGGGAGCCGAACCCGAGGTGGGAGAGAAGAAGGAGGAAGCAGAGAGGCTGA     343
Reported    CTGCAGAGGAGGGAGCCGAACCCGAGGTGGGAGAGAAGAAGGAGGAAGCAGAGAGGCTGA     1080
            ************************************************************

Normal      CCACGGAGGAGCAGGAGAAGGTGGCCGTGCCAGAGGGGGAGAAAGTAGAGACCCCCGGAG     403
Reported    CCACGGAGGAGCAGGAGAAGGTGGCCGTGCCAGAGGGGGAGAAAGTAGAGACCCCCGGAG     1140
            ************************************************************

Normal      TGGATAAGGAGGGTGAAAAAGAAGAGCCGCAGTCGGAGAAGGTGTCAAAGCAAGGGCTGC     463
Reported    TGGATAAGGAGGGTGAAAAAGAAGAGCCGCAGTCGGAGAAGGTGTCAAAGCAAGGGCTGC     1200
            ************************************************************

Normal      CAGCTGAGAAGACACCTTCACTCTGTCCAGAGCTGACAACAGATGATGCCAGACCCCTGA     523
Reported    CAGCTGAGAAGACACCTTCACTCTGTCCAGAGCTGACAACAGATGATGCCAGACCCCTGA     1260
            ************************************************************

Normal      GCAGGCTAAGCAAAGCCAGCAGCCGAGCCAGGTCAGACGATCTAACCGTATGA-------     576
Reported    GCAGGCTAAGCAAAGCCAGCAGCCGAGCCAGGTCAGACGATCTAACCGTATGAAGTGACG     1320
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Fig. 2. Sequence alignment of reported sequence of GJA8 exon 2 retrieved from NCBI (GenBank NG_016242.1) and normal sequence. Star indicates the matches.

This reported sequence was also compared with the sequence of patient sample, which displayed a single nucleotide variation in the coding region of GJA8 exon 2 at nucleotide 1104 (Highlighted in Figures 3 and 4). Amino acid sequence alignment of patient sample with reported sequence revealed that a novel 1104G>C (pE368Q) (GenBank KY556641) point mutation that substitutes glutamic acid, at position 368 with glutamine in patient sample A14 (Fig. 5). Substitution of glutamic acid to glutamine at position 368 in GJA8 exon 2 is a novel mutation as it is not previously reported.

Bioinformatics analysis

I-TASSER anticipated secondary structure of the wild type GJA8 protein. Secondary structure of wild type protein from amino acid 360 to 380 is presented in Figure 6.

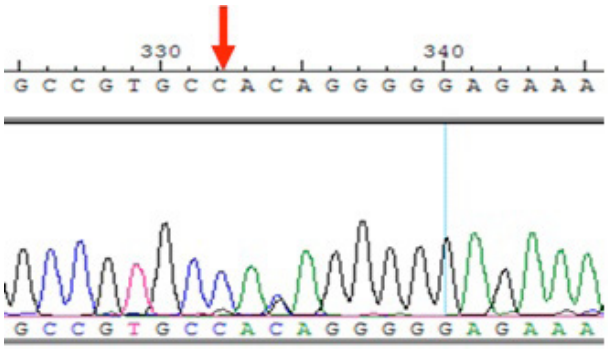

Fig. 3. Sequence Chromatogram of patient sample A14, at reference position 330 to 340. Red arrowhead indicates the position of mutation.

```
Reported    TCTCCTCCATCCAGAAAGCCAAGGGCTATCAGCTCCTAGAAGAAGAGAAAATCGTTTCCC    840
Affected    ---------------------------GNNNAATGCTCTGAGAGAGAAAATCGTTTCCC    32
                                       ******************

Reported    ACTATTTCCCCTTGACCGAGGTTGGGATGGTGGAGACCAGCCCACTGCCTGCCAAGCCTT    900
Affected    ACTATTTCCCCTTGACCGAGGTTGGGATGGTGGAGACCAGCCCACTGCCTGCCAAGCCTT    92
            ************************************************************

Reported    TCAATCAGTTCGAGGAGAAGATCAGCACAGGACCCCTGGGGGACTTGTCCCGGGGCTACC    960
Affected    TCAATCAGTTCGAGGAGAAGATCAGCACAGGACCCCTGGGGGACTTGTCCCGGGGCTACC    152
            ************************************************************

Reported    AAGAGACACTGCCTTCCTACGCTCAGGTGGGGGCACAAGAAGTGGAGGGCGAGGGGCCGC    1020
Affected    AAGAGACACTGCCTTCCTACGCTCAGGTGGGGGCACAAGAAGTGGAGGGCGAGGGGCCGC    212
            ************************************************************

Reported    CTGCAGAGGAGGGAGCCGAACCCGAGGTGGGAGAGAAGAAGGAGGAAGCAGAGAGGCTGA    1080
Affected    CTGCAGAGGAGGGAGCCGAACCCGAGGTGGGAGAGAAGAAGGAGGAAGCAGAGAGGCTGA    272
            ************************************************************

Reported    CCACGGAGGAGCAGGAGAAGGTGGCCGTGCCAGAGGGGGAGAAAGTAGAGACCCCCGGAG    1140
Affected    CCACGGAGGAGCAGGAGAAGGTGGCCGTGCCACAGGGGGAGAAAGTAGAGACCCCCGGAG    332
            ********************************* ****************************

Reported    TGGATAAGGAGGGTGAAAAAGAAGAGCCGCAGTCGGAGAAGGTGTCAAAGCAAGGGCTGC    1200
Affected    TGGATAAGGAGGGTGAAAAAGAAGAGCCGCAGTCGGAGAAGGTGTCAAAGCAAGGGCTGC    392
            ************************************************************

Reported    CAGCTGAGAAGACACCTTCACTCTGTCCAGAGCTGACAACAGATGATGCCAGACCCCTGA    1260
Affected    CAGCTGAGAAGACACCTTCACTCTGTCCAGAGCTGACAACAGATGATGCCAGACCCCTGA    452
            ************************************************************

Reported    GCAGGCTAAGCAAAGCCAGCAGCCGAGCCAGGTCAGACGATCTAACCGTATGAAGTGACG    1320
Affected    GCAGGCTAAGCAAAGCCAGCAGCCGAGCCAGGTCAGACGATCTAACCGTATGAAACACAA    512
            ************************************************************

Reported    CCAAA    1325
Affected    -----    512
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Fig. 4. Sequence alignment of Patient sample with the reported sequence of GJA8 exon 2 submitted in NCBI (GenBank KY556641) and normal sequence retrieved from NCBI (GenBank NG_016242.1). Star indicates the matches. Red arrowhead indicates the substitution in coding region of GJA8 exon 2 at nucleotide 1104.

```
Reported    EQPLGEIPEKSLHSIAVSSIQKAKGYQLLEEEKIVSHYFPLTEVGMVETSPLPAKPFNQF  300
Affected    ----------------------------------------MVETSPLPAKPFNQF  15
                                                    ***************

Reported    EEKISTGPLGDLSRGYQETLPSYAQVGAQEVEGEGPPAEEGAEPEVGEKKEEAERLTTEE  360
Affected    EEKISTGPLGDLSRGYQETLPSYAQVGAQEVEGEGPPAEEGAEPEVGEKKEEAERLTTEE  75
            ************************************************************

Reported    QEKVAVPEGEKVETPGVDKEGEKEEPQSEKVSKQGLPAEKTPSLCPELTTDDARPLSRLS  420
Affected    QEKVAVPQGEKVETPGVDKEGEKEEPQSEKVSKQGLPAEKTPSLCPELTTDDARPLSRLS  135
            *******:****************************************************

Reported    KASSRARSDDLTV       433
Affected    KASSRARSDDLTV       148
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Fig. 5. Amino acid sequence alignment of the patient sample with reported sequence. Difference is highlighted in yellow color which indicates the substitution of glutamic acid to glutamine at codon position 368.

Glutamic acid at point 368 is involved in formation of coiled secondary structure with a total conf. score of 7. Five 3-D models of wild type GJA8 protein on the basis of energy and functional annotation were projected by I-TASSER. PROVE and ERRAT has verified one out of five predicted models of the I-TASSER by giving overall quality factor of 89.880 (Fig. 7). Ramachandran Plot of wild type GJA8 indicates that maximum residues fall in "Highly allowed region" and few in forbidden region (Fig. 8). Superimposed model of wild type and mutated GJA8 was prophesied by SWISS MODEL (Fig. 9), shows no major effect of mutation on protein structure. Stability of wild-type protein is 541.85 kcal/mol and 1104G>C (pE368Q) mutation imparts protein a little stable confirmation that is 539.13 kcal/mol. So the energy difference between mutated and wild type protein is -2.72 kcal/mol.

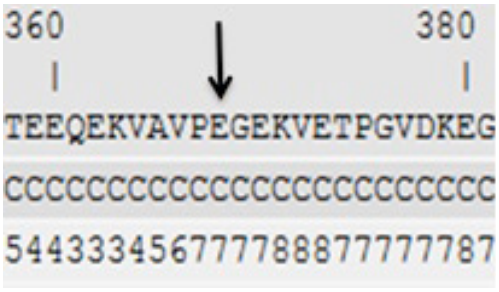

Fig. 6. Secondary structure of wild type GJA8 from amino acid 360 to 380 by I-Tasser. In wild type protein glutamic acid at position 368 as indicated by arrow head is involved in coil formation with a total conf. score of 7. Greater score indicates maximum accuracy of this secondary structure.

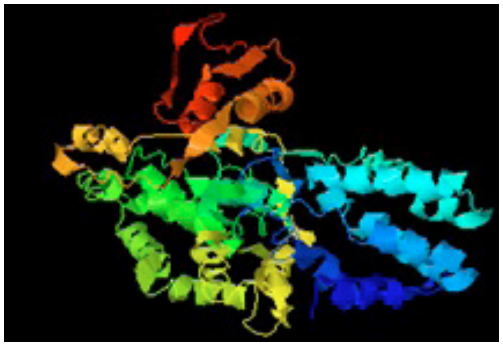

Fig. 7. 3-D structure of wild type GJA8 proposed by I-Tasser with reference to 10 most significant threading templates selected on the basis of their Z-score, with a total quality factor of 89.880 calculated by ERRAT.

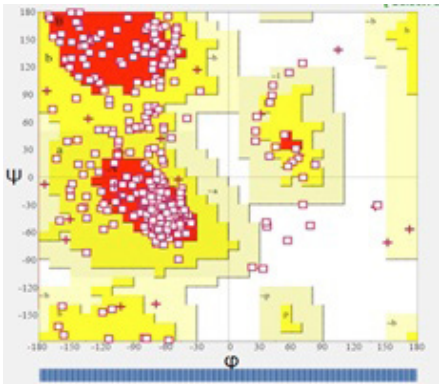

Fig. 8. Ramachandran plot of wild type GJA8 protein, showing most of the amino acid residues fall in the permitted regions, whereas few fall in the prohibited regions of plot.

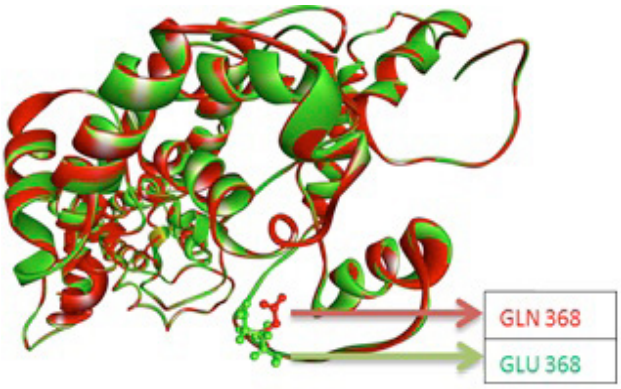

Fig. 9. Superimposed 3-D structure of wild type and mutant GJA8 proteins proposed by SWISS MODEL. Green arrow indicates glutamic acid at position 368 in wild type protein whereas red arrow indicate the position of glutamine in mutant GJA8 protein.

Functional analysis

Impact of mutation on the function of GJA8 protein was determined by online tool HOPE. Each amino acid has its own precise individuality which is allocated by presence of specific charge, size of side chain and hydrophobicity assessment. Conversion of glutamic acid to glutamine neutralizes the negative charge of amino acid at position 368; this loss of charge will affect its interaction with other amino acids and neighboring molecules. Gain and loss of function was estimated by MutPred which provide imperative analysis of mutated residue. This mutation would result in increased number of mutated GJA8 sheets, besides this gain of glycosylation at lys371, gain of methylation at lys371, loss of ubiquitination at lys363 and loss of loop are ultimate consequences of this mutation.

DISCUSSION

In vertebrates the crystalline lens is a biconvex transparent structure, which helps light to focus on the retina. The disruption of proteins results in opacification of the lens, which can result in blindness. The lens consists of: the lens capsule, the epithelial cells and the lens fibres (Beyer *et al*., 2013). Cells present on the surface of lens are metabolically active and sustain cell to cell correspondence to perpetuate transparency of lens (Hejtmancik, 2008). Gap junctions, made up of connexons; sustain the integral function of cells by permitting communication between them. Each connexon comprises a pairs of Connexin43, 46 and 50 subunits (Santana and Waiswo, 2011; Beyer and Berthoud, 2014). They adhere to cell surface; provide anchorage to extracellular matrix, sandwiched between neighboring cells. This facilitates passage of solutes, ions and molecules between cells to maintain proper functioning of avascular organ (Beyer and Berthoud, 2014).

Connexin50 has vast chronicles of reported mutations. At present 34 mutations have been identified which lead to different morphological states (Chen *et al*., 2015; Sellitto *et al*., 2004). These mutation lead to modify secondary and tertiary structure of coded proteins, which ultimately stemmed in its misfolding, unfolding or aggregation (Raju and Abraham, 2011). GJA8 gene code for connexin-50, its expression is exceedingly high in fiber cells, and crucial for maintenance of lens appropriate structure and function (Rong *et al*., 2002).

Recently, GJA8 gene was knocked down in a rabbit model by aid of CRISPR/Cas9 system at zygote level which revealed the significance of GJA8 in perpetuation of lens normal phenotype (Yuan *et al*., 2016). GJA8-/+ mice disclosed phenotype analogous to humans. This revealed the prominence of GJA8 in preservation of eye standard anatomy and precision of CRISPR/Cas9 system as gene editing toll (Yuan *et al*., 2016).

To acknowledge above data, we screen GJA8 gene of 27 cataract patients with no family history of cataract, a subtle 1104G>C (pE368Q) (GenBank KY556641) transversion that substitutes glutamic acid to glutamine was identified at exon 2 of GJA8 gene in one of the patient, which revealed that glutamic acid at position 368 in normal GJA8 protein was changed to glutamine, which is highlighted in Figure 6. Contemporarily missense mutations at 264C>A, 131T>C and 829C>T, in the coding region of GJA8, cause p.P88T, p.V44A and p.H277Y alterations identified respectively in recent years (Ge *et al*., 2014; Zhu *et al*., 2015; Chen *et al*., 2015).

Glutamic acid is a negatively charged amino acid whereas glutamine is neutral. Glutamic acid is decidedly conserved amino acid at this point which accentuate on its functional significance. Amino acid extant from 334 to 385 codes for a chaperon named as “ASF1 like histone chaperone” which implicate proper folding of protein to facilitate its regular action. Substitution of negatively charged amino acid with neutral interrupts its interaction with neighboring molecules but have no inauspicious effect on the overall structure of mutated GJA8 protein.

Energetically mutated GJA8 is immensely stable in comparison to wild type GJA8 with an energy difference of -2.72 kcal/mol. Although functional analysis exhibit miscellaneous posttranslational deformities, which lead to, gain of glycosylation and loss of ubiquitination at some peculiar amino acid residues. Besides this, loss of loop at the site of mutation and increase in number of sheets in overall structure of protein might disrupt systematic folding of protein, which ultimately misfold it and disorder the ordered association of lens cells, which might lead to cloudiness of lens.

CONCLUSION

Mutational screening of GJA8 gene showed substitution of glutamic acid to glutamine at codon position 368 in the coding region of GJA8 exon 2, which is a novel mutation. The extent to which this change interferes with the normal functioning of the protein is not yet known, although it is hypothesized that this region codes for a chaperone which is actually meant for proper folding of protein. Disruption in charge, at extremely conserved site may disturb its tertiary structure to the extent of genesis of cataract. Further functional analysis of this mutation on fiber cell development would illuminate our knowledge with the reasons involved in disruption of ion channels and metabolic inequity in these cells, so that we can get a better view of this communal pathogenesis of lens and this would finally pave paths to enterprise possible genetic and physical therapies.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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