Molecular Genetic Analysis of Congenital Hereditary Cataract

A Thesis submitted to the University of Hyderabad for the award of

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Degree in Department of Biochemistry, School of Life Sciences, University of Hyderabad. Andhra Pradesh (India).

By

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Dedicated to my beloved mother

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DECLARATION

I, Surya Prakash Goud Ponnam, hereby declare that this thesis entitled, "**Molecular Genetic Analysis of Congenital Hereditary Cataract**", submitted by me under the guidance and supervision of Dr. Chitra Kannabiran and Prof. T.Suryanarayana, is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this university or any other university for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled "**Molecular Genetic Analysis of Congenital Hereditary Cataract**" is a record of bonafide work done by Surya Prakash Goud Ponnam, a research scholar at L.V.Prasad Eye Institute, Hyderabad, India and is registered for Ph.D. programme in the Department of Biochemsitry, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or in full to this or any other university or institution for the award of any degree or diploma.

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Surya Prakash G.P.

Abbreviations

µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
A	:	Adenine
APS	:	Ammonium persulfate
bp	:	Base pair
BSA	:	Bovine serum albumin
C	:	Cytosine
c.DNA	:	Complementary DNA
Del	:	Deletion
DMEM	:	Dulbecco's modified Eagles medium
DMSO	:	Dimethylsulphoxide
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxy nucleoside triphosphates
EDTA	:	Ethylene diamine tetraacetic acid
FBS	:	Fetal bovine serum
fs	:	Frameshift
G	:	Guanine
IF	:	Immunofluorescence
Ins	:	Insertion

kDa	:	Kilo Dalton
MCS	:	Multiple cloning sites
ml	:	Millilitre
nm	:	Nanometer
OD	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
RE	:	Restriction enzyme
RFLP	:	Restriction fragment length polymorphism
RNase A	:	ribonuclease A
rpm	:	Revolutions per minute
SDS	:	Sodium dodecylsulfate
SNP	:	Single nucleotide polymorphism
SSCP	:	Single strand conformation polymorphism
T	:	Thymine
TBE	:	Tris borate EDTA
TEMED	:	Tetramethylethylenediamine
TAE	:	Tris Acetic acid EDTA
VA	:	Visual acuity

Contents

Chapter 1

Chapter 1: Introduction and Literature review

1. Human lens:

The lens is also known as the *aquula* (Latin, *a little stream*, dim. of *aqua*, *water*) or *crystalline lens*. Lens is a transparent, biconvex structure in the eye that along with the cornea refracts light to aid in focusing the image onto the retina. This is achieved by changing shape resulting in increase or decrease of the focal length, so that objects at various distances can be focused thereby allowing a sharp image of the object to be formed on the retina. This adjustment of the lens is known as "accommodation".

Figure 1.1.

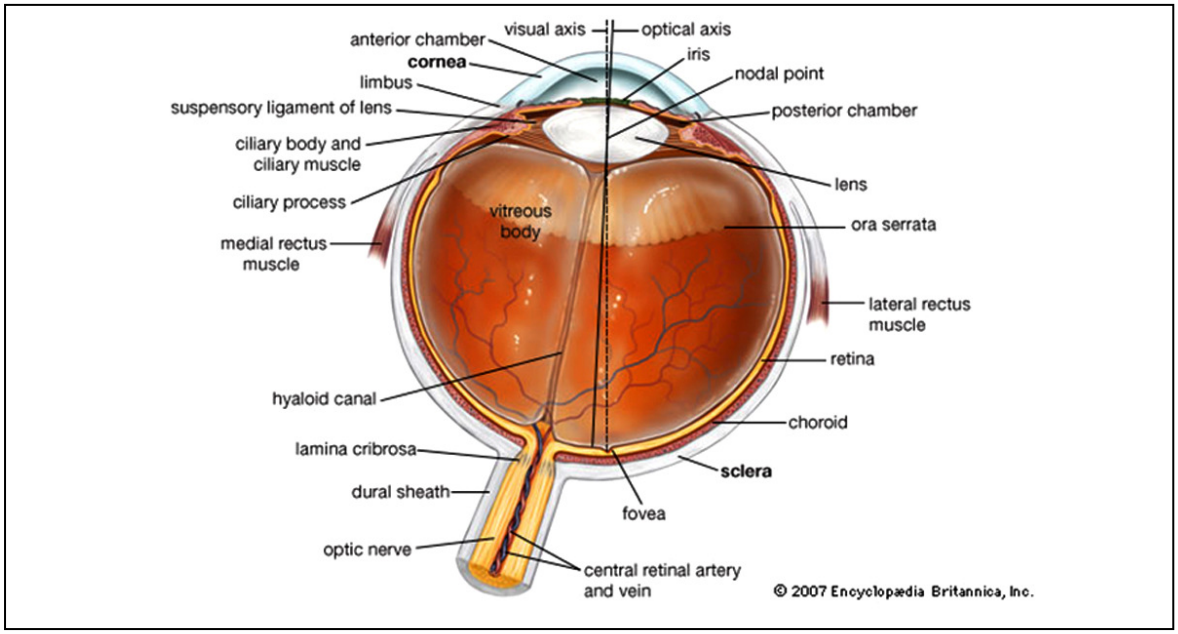


Figure 1.1: The structure of the human eye.

(Source-http://media-2.web.britannica.com)

1.1. Position, Shape & Size

The lens is located in the anterior segment of the eye (Fig.1.1). Towards the anterior side of the lens is the iris, which regulates the amount of light entering into the eye and on the posterior side is the vitreous body, which, along with the aqueous humour on the anterior surface, bathes the lens. The lens is suspended by the zonular fibers, which attach to the lens near its equatorial line and connect the lens to the ciliary body. The lens has an ellipsoid, biconvex shape. The anterior surface is less curved than the posterior. Unlike other organs, lens continues to grow at a very slow pace throughout life. In humans, at birth it weighs about 65 mg, by the first year it is 130 mg and the weight slowly rises thereafter to about 250 mg at 90 years (Duke-Elder 1963; Duke-Elder 1969; Hogan 1971). The diameter of the adults lens varies from 8.8 to 9.2 mm (nearly 10 mm) and has an axial length of about 4 mm but the antero-posterior thickness changes with accommodation (John Forrester 1996). The lens is also known to thicken by 0.023 mm/year. The refractive power of the human lens is roughly one-third of the eye's total power, i.e. approximately 18 dioptres. The refractive index of the lens varies from approximately 1.406 in the central layers down to 1.386 in less dense cortex of the lens (Hecht 1987; Hejtmancik 1995). This index gradient enhances the optical power of the lens and enables it to correct for spherical aberration of focussing. The lens is enclosed within a thin basement membrane called the capsule which helps keep the ocular lens separated from other cells and tissues. Immediately behind the anterior capsule is a single layer of epithelial cells with a diameter of about 13 µM. The lens fibre cells are produced by the mitosis of epithelial cells in the pre-equatorial zone, which elongate and undergo differentiation and then eventually lose all the cell organelles and the nucleus. As the lens fibres elongate and new ones form, the older ones are pushed towards the depth of the

lens so that the youngest lens fibres are the most superficially located. In contrast, the most superficial part of the lens is the youngest or 'cortex'. The substance of the lens can be divided into two distinct divisions. They are the cortex and the nucleus. The nucleus consists of densely compacted lens fibres and has a higher refractive index than that of the cortex. The cortex is the outermost layer of the lens which is seen as zones that are alternatively dark and bright on oblique illumination with a slit-lamp, depending on the propensity to scatter light (Ramanjit Sihota 2003). Successive nuclear zones are laid as development proceeds, and depending on the period of formation, are the embryonic, *foetal*, *infantile*, and *adult nucleus* (corresponding to the lens in early adult life), and finally and most peripherally, the cortex consisting of the youngest fibres (Figure 1.2.)

Figure 1.2.

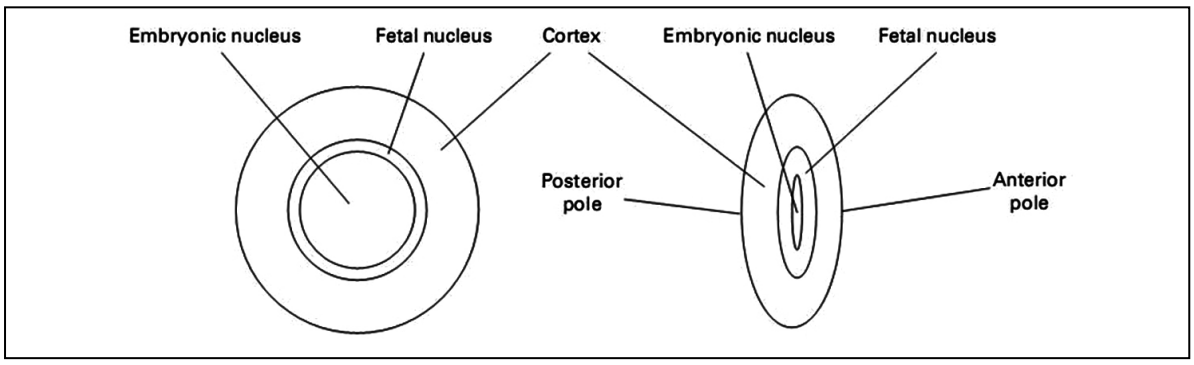


Figure 1.2: The human crystalline lens (Francis, Berry et al. 2000).

Figure 1.3.

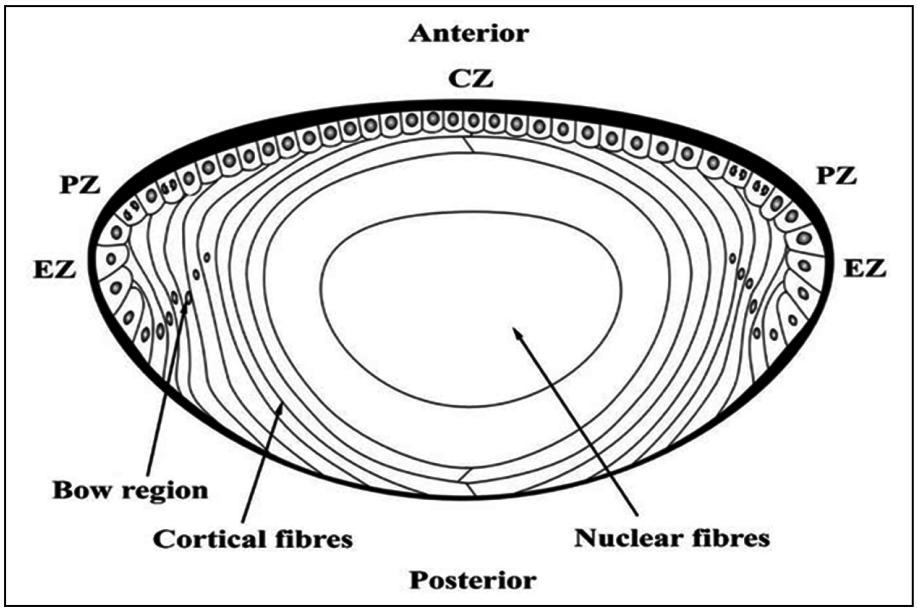


Figure 1.3: Schematic drawing of adult human lens showing the central zone (CZ), pre-equatorial zone (PZ) and equatorial zone (EZ) (Gupta, Johar et al. 2004)

1.2. Development & growth

Development of the human lens begins at the 3 mm embryo length or age 1 week, Unlike the rest of the eye, which is derived mostly from the neural ectoderm, the lens is derived from the surface ectoderm. The first stage of lens differentiation takes place when the optic vesicle, which is formed from outpocketings in the neural ectoderm, comes in proximity to the surface ectoderm (Figure 1.4a). At the 4 mm embryo stage or age 2 weeks, the optic vesicle (OV) induces nearby surface ectoderm to form the lens placode (LP) (Figure 1.4b). The lens placode is a single monolayer of columnar cells.

As development progresses, at the 10 mm stage or the 4th week of embryo, the lens placode begins to deepen and invaginate (Figure 1.4c). As the placode continues to deepen, the opening to the surface ectoderm constricts and the lens cells forms a structure

known as the lens vesicle. By the 10 mm stage, the lens vesicle has completely separated from the surface ectoderm (Figure 1.4d). After the 10 mm stage, signals from the developing neural retina induces the cells closest to the posterior end of the lens vesicle begin to elongate toward the anterior end of the vesicle. These elongating cells eventually fill in the lumen of the vesicle to form the primary fibres, which become the embryonic nucleus in the future (Figure 1.4e.)

Figure 1.4(a-f).

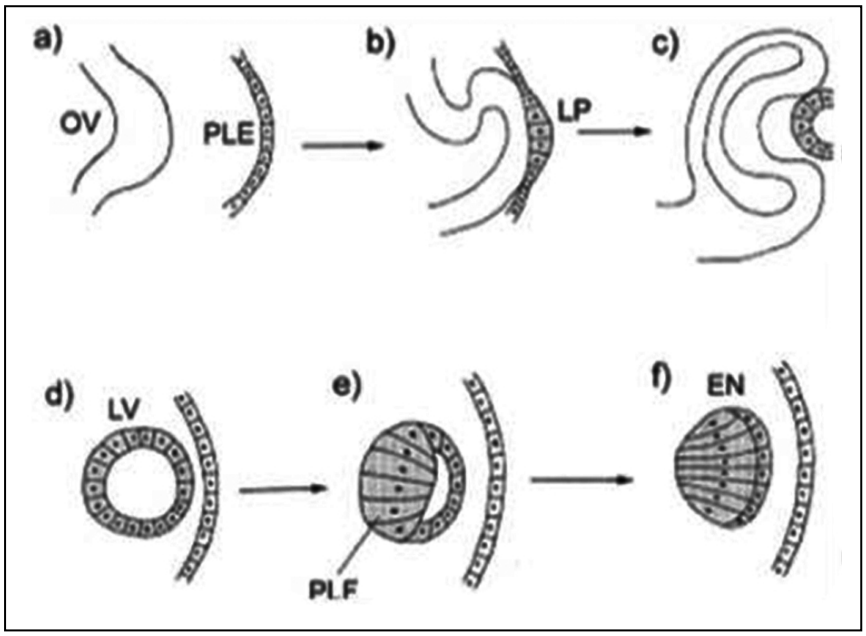


Figure 1.4(a-f): (Reddy, Francis et al. 2004).a: The optic vesicle (OV) approaches the presumptive lens ectoderm (PLE), human age 1week ,3mm embryo length, Carniege stage 4. B: Contact between OV and PLE results in lens placode (LP) formation, human age 2 weeks, 4mm embryo length, Carneige stage 6. C: LP invagination produces a lens pit. D: Lens pit detaches from the surface ectoderm and the lens vesicle (LV) is formed, human age 4 weeks, 10 mm embryo length, Carniege stage 12.e: Posterior lens vesicular cells elongate toward the anterior epithelial cells to form primary lens fibers (PLF). F: the embryonal nucleus is formed following the obliteration of the lens vesicle lumen, human age 6 weeks, 16 mm embryo length, Carniege stage 16.

At 16 mm embryo or 6th week of age, the embryonal nucleus is formed following the obliteration of the lens vesicle lumen (Figure 1.4f). The mature lens epithelial cells are the progenitors of lens fiber cells. Many growth factors and signalling proteins regulate differentiation of lens epithelial cells into fiber cells (Robinson 2004). The cells of the anterior portion of the lens vesicle give rise to the lens epithelium. Additional secondary fibres are derived from lens epithelial cells located toward the equatorial region of the lens. These cells lengthen anteriorly and posteriorly to encircle the primary fibres. The new fibres grow longer than those of the primary layer, but as the lens gets larger, the ends of the newer fibres cannot reach the posterior or anterior poles of the lens. The lens fibres that do not reach the poles form tight, interdigitating seams with neighbouring fibres. These seams are readily visible and are termed sutures shown in figure 1.3 (Brown NAP 1996). The suture patterns become more complex as more layers of lens fibres are added to the outer portion of the lens.

The lens epithelial cells elongate, lose contact with the capsule and epithelium, synthesize crystallin, and then finally lose their organelles as they become mature lens fibres (Andley 2007). From development through early adulthood, the addition of secondary lens fibres results in the lens growing more ellipsoid in shape; after about age 20, however, the lens grows rounder with time. The anterior surface of the lens is lined by single layer of lens epithelial cells (LEC).

1.3. Histology of Lens

The lens is comprised of three main parts: a) the lens capsule b) the lens epithelium and c) the lens fibres.

1.3.1. Lens capsule

The lens capsule forms the outermost layer of the lens. The lens capsule is a non-cellular, thick, smooth, collagenous basement membrane which is transparent. It encloses the lens and anchors the zonular insertions. The capsule varies from 2-28 micrometres in thickness and is usually thickest at the anterior pre-equatorial region and thinnest at the posterior pole. The lens capsule is entirely synthesized by lens epithelium and mainly comprises of Type IV collagen (Dische and Borenfreund 1954), heparin sulphate proteoglycan laminin (Hughes, Laurent et al. 1975), entactin, and fibrectin (Hejtmancik JF 1995). The capsule is very elastic and so causes the lens to assume a more globular shape when not under the tension of the zonular fibres, which connect the lens capsule to the ciliary body.

1.3.2. The lens epithelium

The cells of the lens epithelium are located beneath the lens capsule and are found only on the anterior side of the lens. It is a single layer of cells lining the capsule. It comprises of simple cuboidal epithelium (Shiels and Hejtmancik 2007). Based on its location the lens epithelium is divided into central zone (CZ), pre-equatorial zone (PZ) and equatorial zone (EZ) (Figure 1.3.). Generally the cells of CZ are mitotically quiescent while cells of PZ are proliferative and produce new cells that migrate towards the EZ where they terminally differentiate to form fiber cells. The cells are interconnected by gap junctions and desmosomes and not by tight junctions or zona occludens, unlike typical epithelial cells. Ions of low molecular weight can be exchanged. The epithelium contains Na-K-ATPase and a calmoudlin dependent Ca^{2+}-activated ATPase for the active transport of electrolytes and maintains osmomolarity. There is also an active transport mechanism

for amino acids. The lens epithelium secretes the lens capsule and regulates the transport of metabolites, and electrolytes to the lens fibres.

1.3.3. Lens fibres

The lens fibres are produced by the mitosis of epithelial cells in the pre-equatorial zone, which elongate and undergo differentiation with pyknocytosis and eventually loss of cell organelle and the nucleus. The loss of all intracellular membrane bound organelles is considered to be the terminal stage of differentiation for the fiber cells (Beebe, Vasiliev et al. 2001). Lens fibres are long, thin transparent cells, properly arranged with the diameter typically varying between 4-7 mm and lengths up to 12 mm. The lens fibres stretch lengthwise from the posterior to the anterior poles and are arranged in concentric layers. The middle of the fibres is at the equator. These tightly packed layers of lens fibres are referred to as laminae. The lens fibres are linked together via gap junctions and interdigitations of the cells that resemble “ball and socket” forms.

The lens is held in place by the ‘suspensory ligament’ or ‘Zonule of Zinn’ (Ramanjit Sihota 2003). The transparency of the lens is maintained by the regular arrangement of the lens fibres which are devoid of organelles.

1.4. Nourishment

In humans, by the ninth week of gestation, the lens is surrounded and nourished by a net of vessels, the tunica vasculosa lentis, which is derived from the hyaloid artery. Beginning from the early fourth month of development the hyaloid arteries and related vasculature begins to atrophy and completely disappear by birth. Active transport takes place between the lens and the aqueous owing to the activity of the sub-capsular

epithelium. After birth the lens acquires its nutrients from the aqueous humour by diffusion through the anterio-posterior poles and the Na-K-ATPase present in the equatorial portion of the lens epithelium (Candia 2004).

Glucose is the primary source of energy for the lens which has a respiratory quotient (CO_2/O_2) equal to 1 (Ramanjit Sihota 2003) suggesting that carbohydrate is the chief source of energy. Approximately 80% of the glucose is metabolized via anaerobic respiration as mature lens fibres do not have mitochondria, The lack of aerobic respiration means that the lens consumes very little oxygen (Bloemendal 1981).

1.5. Biochemistry

Water is the major component of the lens. The water content of the human crystalline lens nucleus is 63.4% (S.D.± 2.9%) and cortex is 68.6% (S.D.± 4.3%) (Fisher and Pettet 1973). Neither the total water content of the cortex nor that of the nucleus show any significant change with age (Van Heyningen 1972). Lens contains about 35% by weight of structural proteins, Crystallins, with concentrations reaching up to 450 mg/ml are packed in very high densities in the lens fibres (Bloemendal, de Jong et al. 2004).

The total lens proteins have been subdivided into a) the water-soluble crystallins and b) water insoluble fraction, formerly designated as albuminoid by C.T.Morner in the year 1894 (Hejtmancik 2001). Crystallins are the major water soluble lens proteins and account for almost 90% of the total proteins of the lens fibre cells. Crystallins are essential for maintenance of lens transparency and refraction (Delaye and Tardieu 1983).

Eleven crystallin genes encode over 95% of the water-soluble structural proteins present in the crystallin lens, representing more than 30% of its mass and accounting for its optical transparency and high refractive index (Mackay, Andley et al. 2004). The structural stability of crystallins is important to lens transparency, since they last a lifetime with virtually no protein turnover (Hejtmancik 1998). Crystallins interact to generate a gradient of refractive index so that light can focus to the retina with minimal scattering. Also protein interactions enhance protein stability or solubility. The vertebrate crystallins are divided into two families: α-crystallins and βγ-crystallins according to their elution on gel exclusion chromatography (Morner 1984). The α-, β-, or γ-crystallin types contribute to 40%, 30% and 25% respectively in humans (Graw 1997). Vertebrate lens βγ-crystallins have two domains, each comprising two structural motifs with signature folds called 'Greek key motifs' (Piatigorsky 2006). The Greek key motifs are so-called because they resemble a common element in Greek pottery and consists of four–stranded antiparallel β-sheet structures. The relative position of the two domains in γB- and βB2-crystallins (γB and βB2) differs in crystallographic structures, having either ‘closed’ or ‘opened’ conformation, respectively (Hejtmancik, Wingfield et al. 2004) i.e. in homodimeric βB2-crystallin, the extended conformation of the connecting peptide has been suggested to force the βB2-molecule to favor intermolecular domain interactions compared with intramolecular contacts in monomeric γB-crystallin. However, in monomeric γB, intra-molecular domains form the interface, whereas, in homodimeric βB2, the interface consists of residues from the N-terminal domain of one monomer and residues from the C-terminal domain of the second monomer in a switched domain fashion (Mayr, Jaenicke et al. 1994). βγ-Crystallins are more heterogeneous with seven subunits (four acidic βA1-, βA2-, βA3-, and βA4-crystallin, and three basic βB1-, βB2-,

and βB3-crystallin) in the hetero-oligomeric β-crystallin and six subunits (γA-γF-crystallin) in the monomeric γ-crystallin (Lampi, Ma et al. 1997). Besides their presence in the lens crystallins, they are found outside the vertebrate eye lens. αB-crystallin expression is found to be in the retina, heart, skeletal muscles, skin, brain and other tissues (Bhat and Nagineni 1989; Dubin, Wawrousek et al. 1989; Klemenz, Frohli et al. 1991). Kato *et al.* 1991 had reported the expression of αA-crystallin, by immunoassay method, in spleen at very low concentrations besides its significant expression in the lens (Kato, Shinohara et al. 1991). βB2-crystallin is expressed ubiquitously and is found in high levels in the brain (Ganguly, Favor et al. 2008), retina (Liedtke, Schwamborn et al. 2007), testis (Magabo, Horwitz et al. 2000), where as βB2-crystallin transcript was not found in rat lung, thymus, kidney and liver or in human brain and testis (Magabo, Horwitz et al. 2000). Sinha and co-workers (Sinha, Klise et al. 2008) have reported βA3/A1-crystallin polypeptides only in the astrocytes of the nueral retina and have demonstrated that βA3/A1-crystallin is an important regulatory factor mediating vascular patterning and remodelling during development in astroglial cells of the retina.

1.6. The lens: transparency and cataract

Transparency of the lens is due to the orderly arrangements of the lens fibres such that the incoming light source (stimuli) is refracted and is focused on to the retina. To achieve high transparency and high refractive index the lens need to have high concentrations of proteins in it. The lens fiber cells adopt a flattened hexagonal profile that facilitates their packing into an ordered array such that the spaces between the cells are smaller than the wavelength of light. The lens maintains this precise cellular architecture and prevents light-scattering by controlling the volume of its constituent cells, preventing dilation of

the normally narrow extracellular space, and maintaining the solubility of lens crystallins to stop their aggregation. In late nineties liquid-like order was proposed to be the basis of transparency of the ocular tissues (Benedek, Pande et al. 1999). Although widely debated, recent investigations on understanding the maintenance (Donaldson, Musil *et al.* 2010) of the lens transparency has focused on circulation model proposed by Donaldson *et al.* 2001 (Donaldson, Kistler et al. 2001) which postulates that there is an existence of an ionic flow from one cell to another through the lens fibre cell cytoplasm which generates a current and a flow of solutes and fluid. This model was based on the location and activities of different types of transporters in the lens.

Cataract is defined as any opacity on the lens which impairs vision. Cataract is caused by the degeneration and opacification of the lens fibres already formed or the formation of aberrant lens fibres or deposition of any other material in their place. Hereditary cataracts are clinically and genetically heterogeneous, often presenting as congenital or developmental cataracts that arise at birth or during the first decade of life. As these opacities can cause blurring of vision during development by interfering with the sharp focus of light on the retina they may result in failure to establish appropriate visual cortical synaptic connections with the retina, and result in permanent visual loss.

There are different models of cataractogenesis that have been put forward. One is that cataract is a protein folding disease. The hypothesis of a protein folding disease holds that cataract may result from an insufficiency in the protein chaperoning system of the cell. A protein folding disease would usually result when the unfolded proteins accumulate faster than the chaperoning system can deal with aggregating protein (Dobson 1999; Csermely

2001). Lack of the ability of the mature lens fibre cell to mount a cellular stress response and the use of a prominent lenticular structural protein, α-crystallin, functioning as a chaperone is the basis for considering cataract as a protein folding disease. As the heat shock response attenuates with ageing the capacity of ageing cells to deal with unstable proteins thus also decays with age explaining why such a disease is more prevalent in the aged. The prevalent hypothesis for age-related cataract is that, with time, as lenticular proteins unfold and/or become modified and start to unfold, the chaperone capacity of alpha crystallin will be used up and protein aggregates will be formed (Horwitz 1992; Clark and Muchowski 2000).

A second school of thought is that cataracts could result from altered protein interactions. The high level of polydispersity, a measure of the orderly distribution of the crystallins proteins in the lens, among the crystallin components not only reduces the risk of protein crsytallization within the lens but also contributes to an even and dense packing resulting in transparency and high refraction of the lens. Alpha crystallin has a dominating effect on the overall repulsive interaction of the mixture of cryatllins in the lens cytoplasm (Veretout and Tardieu 1989). Changes in solubility or attractive properties can cause cataract without the protein misfolding and before it unfolds. Two studies described two point mutations of surface residues of human gamma crystallins that resulted in reduced protein solubility and were thereby proposed to cause cataract (Heon, Priston et al. 1999; Kmoch, Brynda et al. 2000). The post-translational modifications to which crystallins are subject, could cause alterations in protein interaction that are causally involved in ageing cataract (Bloemendal, de Jong et al. 2004).

1.7. Classification of cataracts

Many classifications schemes have been proposed for cataract (Sparrow, Bron et al. 1986; West and Taylor 1986; Brown, Bron et al. 1987; West, Rosenthal et al. 1988; Laties, Keates et al. 1989; Sharma, Vajpayee et al. 1989; Lambert and Drack 1996; Reddy, Francis et al. 2004). Clinically, cataract may be classified based on: a) morphological characteristics of the cataract such as it location, size and appearance and b) the aetiology of the cataract. Based on the aetiology, cataract can be classified into four broad categories, namely: i) Congenital and developmental cataracts ii) Senile cataracts iii) Acquired cataracts and iv) Secondary (complicated) cataracts (Balasubramanian, Bansal et al. 1993). Since most of them do not quantify the degree of opacity and there is a subjective element involved in accurately diagnosing the extent & phenotype of cataract they cannot be used for cross-sectional and longitudinal research studies. Chylack *et al*., 1989 proposed the Lens Opacities Classification System (LOCS) which was at a later stage modified and referred to as LOCS II and LOCS III respectively (Chylack, Leske et al. 1988; Chylack, Leske et al. 1989; Chylack, Wolfe et al. 1993). LOCS III system uses a set of colour photographs as standards of comparison. This is the most widely accepted classification in the current era. The following features are graded:

a) Cortical cataract (C1-C5)

b) Nuclear Opalescence (NO1-NO6)

c) Nuclear Colour (NC1-NC6)

d) Posterior subcapsular cataract (P1-P5)

In spite of this system being reproducible and reliable to some extent, it also has a subjective element in it. These classifications are based on the time of development, aetiology, location within the lens, colour, texture, shape and degree of opacification.

Reddy et al. 2004 had classified isolated hereditary cataracts based on the position of the opacity within the lens and its appearance on slit-lamp examination (Reddy, Francis et al. 2004).

Figure 1.5.

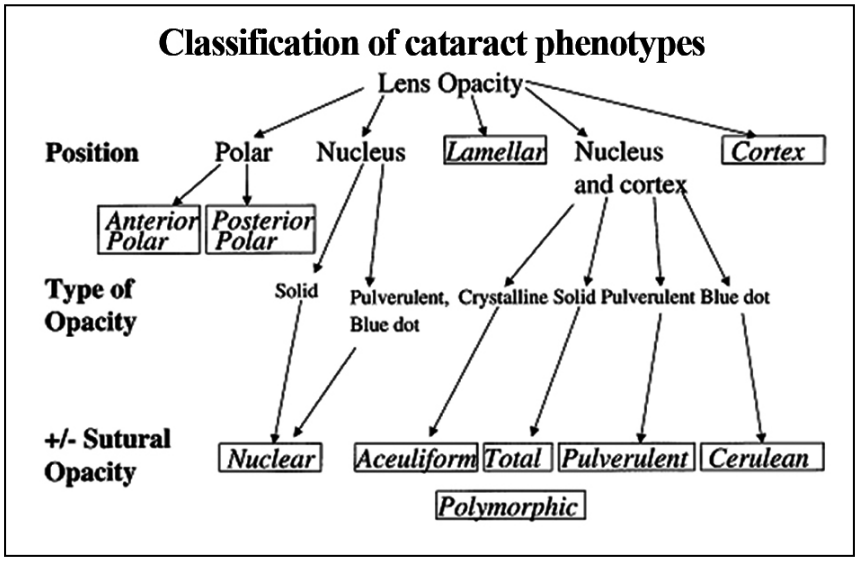


Figure 1.5: Clinical algorithm to aid the classification of phenotyoes in inherited cataract. Source : (Reddy, Francis et al. 2004)

Figure 1.6.

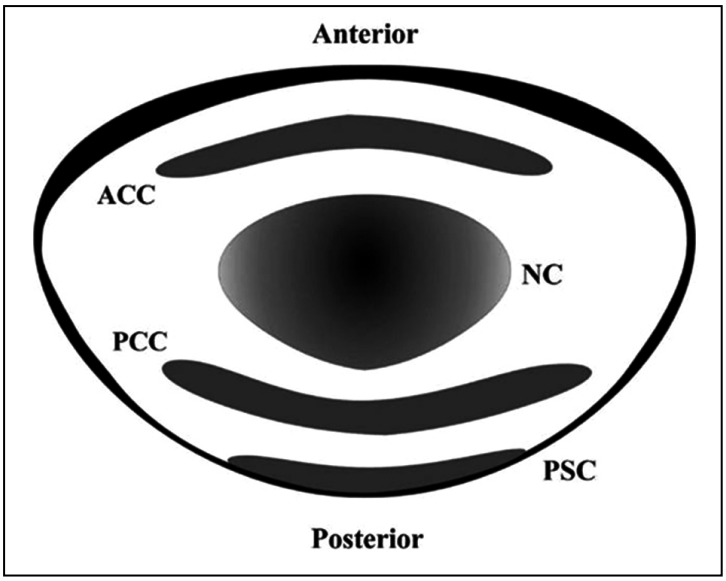


Figure.1.6: Schematic drawing of human lens showing various types of cataracts. Nuclear cataract (NC), anterior and posterior cortical cataracts (ACC and PCC), posterior subcapsular cataract (PSC).

Based on the position of the opacity on the lens hereditary cataracts have been classified into Polar, Nucleus, Lamellar, Cortex (Nuclear and cortex). Polar cataracts have been further subdivided into anterior and posterior polar depending upon the side of the pole at which there is lens opacity (Table 1.1). Based on the appearance or the type of opacity cataracts have been classified into following types (Reddy, Francis et al. 2004; Armstrong 2005). They are:

A. **Pulverulent:** characterized by powdery, dot-like, (pulverized) opacities that may be present throughout the lens.

B. **Aceuliform:** This form of cataract is associated with needle–like projections extending from the nucleus into the anterior and posterior cortex. It has been called "speisskataract" and "needle-shaped cataract" and there may well be overlap between this phenotype and the coral-like protuberances of coralliform cataract.

C. **Cerulean:** This is a distinctive form of lenticular opacification, both in terms of appearance and prognosis. Ceruelan cataracts have a discrete pinhead-shaped blue and white opacities that are distributed throughout the lens becoming more numerous in the cortex where they may fom large cuneiform (wedge-like) shapes in the mid periphery.

D. **Polymorhic:** The polymorphic cataract phenotype was first described in three families with wide variations in grape-like lens opacities that spared the nucleus.The distribution of the opacities varied from the superficial to be the deep layers of the cortex.

E. **Sutural:** Sutural cataracts are characterised by prominent opacification of the anterior and posterior sutures of the lens without other forms of morphological change within the lens.

Table 1.1.

Type of cataract	Position of the opacity in the lens
Anterior polar	Anterior pole
Posterior polar	Posterior pole
Nuclear	In the lens nucleus
Lamellar	Involving specific lamellae
Cortical	Lens cortex
Total	Nucleus+cortex

Table 1.1: Types of cataracts based on the position of the opacity on the lens

1.8. Risk factors associated with cataracts

There are several ongoing epidemiological studies, which have helped to figure out that there are multiple risk factors associated with cataract in adults. Some identified till date are ageing, systemic diseases like diabetes etc., metabolic disturbances, radiation

(Ainsbury, Bouffler et al. 2009), trauma, drugs, low antioxidant intake, cigarette smoking, alcohol, corticosteroids, vitamin deficiencies and genetic factors involving mutations in the various gene products which play a major role in eye lens transparency (Balasubramanian, Bansal et al. 1993; Cumming and Mitchell 1997; Francis, Berry et al. 1999; Hammond, Snieder et al. 2000; McCarty, Nanjan et al. 2000; Harding 2002; Krishnaiah, Vilas et al. 2005). Congenital cataracts may be associated with multitude of factors like harmful radiation, intrauterine infections such as rubella, herpes simplex (Raghu, Subhan et al. 2004) and syphilis, trauma, and steroid or other drug use during pregnancy by the mother. (Johar, Savalia et al. 2004). Metabolic disorders either of maternal or fetal origin are associated with cataract [galactosemia (Ramakrishnan, Sulochana et al. 1998), hyperferritinaemia (Beaumont, Leneuve et al. 1995) etc.)]. Numerous syndromes (Down's syndrome, Wilson's disease, myotonic dystrophies) (He and Li 2000), or other ocular diseases are also associated with congenital cataracts (Wirth, Russell-Eggitt et al. 2002). About one-third to one-fifth of the congenital cataracts has been reported due to mutations in the crystallin genes and the other genes responsible for the structure and function of the lens (Graw 1999; Rahi and Dezateux 2001; Hejtmancik 2008).

1.9. Epidemiology of Cataract

Age-related cataracts are the major cause of visual impairment and blindness throughout the world (Congdon, Friedman et al. 2003; Resnikoff, Pascolini et al. 2004) and they account for almost 42% of the total blindness (Brian and Taylor 2001). With the number of people blind due to cataract increasing by approximately one million people a year is an alarming issue (Foster 1999). The Andhra Pradesh Eye Disease Study (APEDS) conducted by L.V.Prasad Eye Institute, Hyderabad reported that cataract alone contributes to 44% of the total blindness

in India (Dandona, Dandona et al. 2001). Other Indian studies also reported cataract to be the major cause of blindness (Vijaya, Gupta et al. 1997; Murthy, Gupta et al. 2001; Nirmalan, Thulasiraj et al. 2002; Nirmalan, Krishnadas et al. 2003). Cataracts account for almost one-tenth of the total childhood blindness in India (Dandona, Williams et al. 1998). While prevalence of congenital cataracts is 1-6 in 10,000 live births, the incidence is 2.2 to 2.49 per 10,000 live births in Western countries (Graw 1999; Francis, Berry et al. 2000; Reddy, Francis et al. 2004; Resnikoff, Pascolini et al. 2004). Developing countries are likely to have higher prevalence of cataracts resulting from infections, dehydration and trauma (Eckstein, Vijayalakshmi et al. 1996). There is a wide variation in the prevalence of childhood cataracts. The prevalence of cataract blindness in children in developing countries is close to 4 per 10,000 and is ten times that seen in the developed world (Foster, Gilbert et al. 1997) while in India it is 6.5 per 10,000 live births. Nearly half of the entire bilateral cataract and 60% of the unilateral cataracts have no identifiable cause (Merin and Crawford 1971; Apple, Ram et al. 2000). Prevention of visual impairment due to congenital cataract is an important component of the World Health Organization's (WHO) international program for the elimination of avoidable blindness by 2020 (Thylefors 1998). Hereditary cataracts account for almost one-third to one fourth of the childhood cataracts in the Western world (Marner, Rosenberg et al. 1989; Lund, Eiberg et al. 1992; Rahi and Dezateux 2000; Messina-Baas, Gonzalez-Huerta et al. 2006)

1.10. Genetics of Congenital Hereditary Cataracts

Hereditary congenital and early-onset cataracts show Mendelian inheritance. They can be inherited in an autosomal dominant (AD) or autosomal recessive (AR) mode. (Balasubramanian, Bansal et al. 1993; Hejtmancik 1995; Kannabiran and Balasubramanian

2000; Hejtmancik 2008). Nineteen genes are associated with autosomal dominant (AD) and nine genes are associated with recessive inheritance. The majority of studies have dealt with autosomal dominant cataracts (Table 1.2). Earlier literature on congenital cataract linked to the X-chromosome was not convincing and it was thought to be a part of Nance-Horan syndrome (NHS) (Warburg 1989). Fraccaro M *et al*. (Fraccaro, Morone et al. 1967) have linked non syndromic X-linked cataract with the Xg blood group on Xp22. Francis *et al*. (Francis, Berry et al. 2002) examined twenty three members of a four generation family with nuclear cataracts and reported the first isolated, non-syndromic, X linked cataract and linked it to Xp22. Recent reports by Brooks *et al.* (Brooks, Ebenezer et al. 2004) and Craig *et al.* (Craig, Friend et al. 2008) have also described novel cataract phenotypes linked to Xp22.13 and Xq24 respectively. These reports suggest genetic heterogeneity among X-linked congenital cataract pedigrees with associated abnormalities. Coccia M and co-workers (Coccia, Brooks et al. 2009) recently reported that NHS and X-linked cataract are allelic disorders.

Genes that have been identified as being associated with isolated autosomal dominant or recessive congenital cataract can be divided into 5 groups including:

(1) Genes encoding crystallins: *CRYAA*, *CRYAB*, *CRYBA1/A3*, *CRYBA4*, *CRYBB1, CRYBB2*, *CRYBB3, CRYBB4*, *CRYGC*, *CRYGD* and *CRYGS*.

(2) Genes encoding cytoskeleton proteins such as *BFSP1* and *BFSP2*

(3) Genes encoding transcription factors such as *PITX3* and *HSF4*

(4) Genes encoding membrane transport and channel proteins: *GJA3*, *GJA8*, and *MIP* (also known as *AQP0*).

(5) Others: *GCNT*, *CHMP4B, EPHA2* and *GALK1*.

1.11. Crystallins:

1.11.1. α-Crystallins

The α-Crystallins are the most abundant soluble proteins in the lens and play essential roles in maintaining lens transparency. α-crystallins are composed of two gene products: alpha-A and alpha-B, for acidic and basic, respectively. The α-crystallins (αA- and αB-crystallin) comprise up to 40% of the protein mass of the lens, and belong to the small heat shock protein family (sHSPs) and are in a molar ratio of roughly 3:1. These function as molecular chaperones. They facilitate the correct folding of other lens proteins, and are of extreme importance in keeping lens proteins in a functional state (Kumar, Ramakrishna et al. 1999). Post-translational modifications of alpha A-crystallin that were detected included 2 phosphorylated serine residues (one of which appears to be unique to human lenses), deamidation at some glutamine and asparagine residues, a disulfide bond between cysteine-131 and cysteine-142, and loss of the COOH-terminal serine residue. Three phosphorylated serine residues, but no deamidation, were found in alpha B-crystallin (Miesbauer, Zhou et al. 1994). Post translational modifications of the α–crystallins was suggested to decrease the chaperone activity (Miesbauer, Zhou et al. 1994). It has been reported that α-crystallin chains have a propensity to keep their net charge conserved throughout evolution (de Jong, Zweers et al. 1984). In the vertebrate lens, αA-and αB-crystallins form hetero-oligomers of variable size and a quaternary structure that binds and sequesters damaged proteins, preventing the formation of particles that scatter light.

A total of nine mutations in *CRYAA* are reported, seven in autosomal dominant (AD) and two in autosomal recessive (AR) cataract. Ten mutations are known till date in *CRYAB*, nine in AD and one in AR cataract (Listed in Appendix 1, Table 1.2).

1.11.2. β-Crystallins

The ß-crystallin family contains seven proteins (three *CRYBB* and four *CRYBA*). Beta-crystallins form the major constituents of the crystallins in the lens. The gene structure of the β-crystallins is very similar, suggesting that they may have been derived from a single ancestor (Piatigorsky 1987; van Rens, de Jong et al. 1992). The β-crystallins appear in the lens as heterogeneous dimers (ßL) to octamers (ßH). Beta and gamma-crystallins are considered as a superfamily. The most distinct feature of individual ß-crystallin molecules is their N- and C-terminal arms. In addition to sequence variations, the N-terminal arms differ significantly in length (Lubsen, Aarts et al. 1988). The C-terminal arm of the ß-crystallin appears to be responsible for protein–protein interactions within the lens (Norledge, Trinkl et al. 1997). Different β-crystallin proteins are found in both prenatal and postnatal developing lens and their interaction with each other, as well as with other lens proteins, are postulated to be important in maintenance of lens transparency (Bax, Lapatto et al. 1990; Russell and Chambers 1990). β-crystallins form aggregates of different sizes and are able to self-associate to form dimers or to form heterodimers with other beta-crystallins. The β- and γ-crystallins share an extremely stable common core structure comprising four twisted β-pleated sheets termed "Greek key motifs," which are organized into two domains (Wistow, Turnell et al. 1983). In the β-crystallins, individual Greek key motifs are encoded by separate exons. The β-crystallins associate into higher order assemblies whiles the γ-crystallins exist in solution as monomers. Both the β- and γ-crystallins tend to be more highly expressed at early developmental times in elongating fiber cells, so that they are found primarily in the lens nucleus (Aarts, Lubsen et al. 1989). Mutations have been reported in all the β-crystallin genes except *CRYBA2* in congenital hereditary cataracts. Mutations in *CRYBA1/A3, CRYBA4,*

CRYBB1, CRYBB2, CRYBB3, have been reported for autosomal dominant and mutations in *CRYBB1, CRYBB3* are reported for autosomal recessive cataracts (Table 1.2).

1.11.3. γ-Crystallins

γ-Crystallins are a homogeneous group of highly symmetric, monomeric proteins. They are differentially regulated after early development. Five γ-crystallin genes (*CRYGA*, *CRYGB*, *CRYGC*, *CRYGD* and *CRYGS)* and two pseudogenes (*CRYGE* and *CRYGF*) are tandemly organized in a genomic segment as a gene cluster. Only *CRYGC* and *CRYGD* are known to encode abundant lens γ-crystallins in humans (Russell, Meakin et al. 1987; Brakenhoff, Aarts et al. 1990). The γ-crystallins have two domains with each domain composed of two Greek key motifs (Blundell, Lindley et al. 1981). The γ-crystallins are monomeric with a molecular mass of 21 kDa and comprise about 40% of the total proteins in the mouse lens and 25% in the human lens (Wistow and Piatigorsky 1988; Graw 1997). It is this crystallin that is responsible for the so-called "Cold cataract". If the temperature at neutral pH is lowered below 10ºC, γ-crystallin precipitates. γS-crystallin is characterized by an additional α-helix, which is not found in the other crystallins. In lenses of individuals less than two years of age, the fraction of γ-crystallins has been reported to be 35% *CRYGS*, 45% *CRYGC*, and 20% *CRYGD* (Siezen, Thomson et al. 1987). Mutations in *CRYGC* and *CRYGD* have been identified to cause isolated autosomal dominant congenital cataracts (listed in Table 1.2). Mutants Thr5Pro, Trp157X, Arg168Trp, Trp157X and a 5-base insertion mutant of *CRYGC* in human cataract (Fu and Liang 2003; Talla, Narayanan et al. 2006; Talla, Srinivasan et al. 2008); (Talla, Srinivasan et al. 2008) as well as three other murine *CRYGD* mutants (Sandilands, Hutcheson et al. 2002) have been

studied with respect to altered stability, interactions, aggregation and/or solubility of mutant proteins.

1.12. Intermediate filament proteins

Intermediate filament (IF) proteins are suggested to play an important role in supporting cellular architecture and providing mechanical strength to the cell. IFs have an average diameter of 10 nm, which is between that of the actin and microtubules, although they were initially designated “intermediate” since their average diameter was between those of narrower microfilaments (actin) and wider myosin filaments (Ishikawa, Bischoff et al. 1968). Two lens-specific intermediate filament-like proteins, CP49 (also known as phakinin) and CP115 (also known as filensin) are expressed only after fiber cell differentiation is initiated (Quinlan, Carte et al. 1996), but do not accumulate to maximum levels until fiber cell has fully elongated (Blankenship, Hess et al. 2001). These intermediate filament-like proteins are the constituents of the fibre cells of the eye lens. Both proteins are found in a structurally unique cytoskeletal element that is referred to as the beaded filament (BF) (FitzGerald and Gottlieb 1989; Ireland and Maisel 1989). Beaded filament structural protein (BFSP) genes are members of the vertebrate intermediate filament gene superfamily (Rendtorff, Hansen et al. 1998). Beaded filament structural proteins (BFSPs) are highly divergent members of the intermendiate filament protein family and are the major components of the beaded filaments, which are abundant in lens fiber cells. These beaded filament proteins consists of a 7-9 nm backbone filament with 12-15 nm globular protein particles spaced along it (Jakobs, Hess et al. 2000).

During lens development, the anterior ends of the lens fiber cells progressively elongate into the space between the lens epithelium and the embryonic nucleus where as the

posterior ends lie between the posterior lens capsule and embryonic nucleus. Elongation continues unitl the fiber cell ends from opposing directions meet to form the lens sutures. These BPSPs possess structural features and unusual characteristics that distinguish them from canonical IF proteins (Perng and Quinlan 2005). In vitro, CP49 and filensin do not form IFs on their own. In vitro studies suggest that CP49 and filensin have a distinct co-assembly mechanism. Whilst CP49 self-assembles into thick bundles of filaments, filensin only forms short fibrils, but when combined together they form filaments. Beaded filaments are considered to be important in facilitating the chaperone activity of α-crystallin assemblies (Graw 2004). In mice, expression of both *BFSP1* and *BFSP2* proteins are absent in lens epithelial cells and first appear in the anterior end of the fibre cells after fiber cell elongation has begun. There is a gradual transition in the distribution of the beaded filament proteins from being membrane-associated to cytoplasm as the lens matures (Blankenship, Hess et al. 2001). Experiments on targeted homozygous gene knockout of BFSP2*/CP49* protein in mice by Alizadeh and colleagues (Alizadeh, Clark et al. 2002) have demonstrated sharply reduced expression of CP49 protein although filensin mRNA levels appeared unchanged. While direct examination of lenses showed no obvious loss of lens clarity the slit lamp examination revealed the emergence of opacification in even the youngest animals and the opacification worsened with age. Targeted ablation of the BFSP1/ CP115/filensin) successfully blocked the production of filensin mRNA, reduced levesl of filensin's assembly partner CP49, and prevented the assembly of beaded filaments (Alizadeh, Clark et al. 2003). Light microscopic analysis neither showed obvious changes in fetal development nor in the differentiation of epithelial cells into mature fibre cell. Slit lamp examination revealed subtle opacification of the lenses of homozygous null animals appeared at approximately 10 weeks of age and worsened progressively in older animals. Six month old homozygous animals had a cataract

of approximately stage 3 on a scale of 1 to 6. Lenses of the wild type were clear. Heterozygous lenses showed an intermediate phenotype with slight opacification in the older animals.

1.12.1. Beaded filament structural protein 1

Beaded filament structural protein 1 (*BFSP1*) is located on 20p12.1. Ramachandran et al. reported the first mutation in the *BFSP1* gene in a consanguineous Indian family with AR inheritance by mapping the locus and screening of *BFSP1* (Ramachandran, Perumalsamy et al. 2007). Affected individuals had cotton-like cortical opacities with occasional grape like cysts in the anterior cortex, nuclear or nuclear sclerotic cataracts with no other ocular anomaly.

1.12.2. Beaded filament structural protein 2

Beaded filament structural protein 2 (*BFSP2*) gene located on chromosome 3q22.1 with seven exons. *BFSP2* is also referred to as phakinin or CP47, phakosin or CP49, and LIFL-L. Animal experiments in mice suggests that a natural mutation found in the Bfsp2 gene in mouse 129 strain causes the loss of CP49/*Bfsp2* and induces vimentin-dependent changes in the lens fibre cell cytoskeleton (Sandilands, Wang et al. 2004). Similar experiments by Simirskii et al. (Simirskii, Lee et al. 2006) in FVB/N mice obtained from various sources showed them to harbor a 6-kb deletion of the *CP49* gene identical to that previously reported in mouse strain 129 with undetectable levels of CP49 in the lens while the C57BL/6 mice did not have this modification. Animal experiments on mice by Alizadeh and co-workers had suggested that *BFSP2* can regulate beaded filament protein stoichometry (Alizadeh, Clark et al. 2002).

Conley and his co-workers (Conley, Erturk et al. 2000) reported the first human *BFSP2* mutation in a six generation American family with AD mode of inheritance. Till date; four mutations have been reported in the literature (Table.1.2) with a wide range of phenotypes that includes progressive cataract (Conley, Erturk et al. 2000; Zhang, Gao et al. 2006; Cui, Gao et al. 2007), sutural cataracts (Zhang, Guo et al. 2004; Zhang, Gao et al. 2006), nuclear cataracts (Conley, Erturk et al. 2000), cortical opacity, stellate and spoke like cataracts (Jakobs, Hess et al. 2000) etc. The exact mechanism by which the *BFSP2* mutations can give rise to cataracts is still unknown.

1.13. Transcription factors:

1.13.1. MAF (Avian Musculoaponuerotic Fibrosarcoma Oncogene Homolog)

The *MAF* gene on chromosome 16q23.1 encodes Maf, a basic region leucine zipper (bZIP) transcription factor expressed in the lens placode, vesicle and, later, the primary lens fibres (Sakai, Imaki et al. 1997; Yoshida, Imaki et al. 1997; Kawauchi, Takahashi et al. 1999). The Maf family of bZIP transcription factors was first identified through the *v-maf* oncogene, an avian retrovirus transforming gene, and *maf* is the cellular homologue (Nishizawa, Kataoka et al. 1989). *Maf* has two isoforms produced by alternative splicing. The first isoform has 2 exons encodes for 403 amino acids with a molecular weight of 41.96 KDa whereas the second isoform has 1 exon and encodes a protein of 38.49 KDa and 373 amino acids. Both homodimers and heterodimers formed by Maf bind to two known Maf response elements (MAREs), with varying affinities and transactivation potentials (Kataoka, Nishizawa et al. 1993; Kataoka, Noda et al. 1996). MARE elements are found in the promoters of the crystallin genes and *PITX3* (Ogino and Yasuda 1998; Semina, Murray et al.

2000). It is a member of the 'large Maf' family (Blank and Andrews 1997), which also includes c-Maf (Kataoka, Nishizawa et al. 1993), L-Maf which plays an important role in induction and lens differentiation (Ogino and Yasuda 1998), Mafb which is important in hindbrain and otic development in the mouse) (Cordes and Barsh 1994) and NRL which functions in the neural retina (Swaroop, Xu et al. 1992).

The key role of c-Maf protein in eye development is highlighted by the fact that homozygous null mutant mouse embryos exhibit defective lens formation and microphthalmia, and show decreased expression of crystallins (Kawauchi, Takahashi et al. 1999; Kim, Li et al. 1999). Two mutations have been identified in the *MAF* gene with AD mode of hereditary cataracts and are listed in Table 1.2.

1.13.2. Paired-Like Homeodomain Trascription factor 3 (PITX3)

PITX3 is a paired-like homeodomain transcription factor that belongs to the RIEG/PITX homeobox gene family (Semina, Reiter et al. 1997). *PITX3* is located on 10q24.32 and has 4 exons. *PITX3* encodes a 31.8 kDa protein with 302 amino acids. Pitx3 is expressed in the developing lens, skeletal muscle, and dopaminergic (DA) neurons of the substantia nigra in the brain (Smidt, Smits et al. 2004). In mice Pitx3 is expressed in the mesencephalic dopaminerigic (DA) neurons that are located in the ventral midbrain and that forms the susntantita nigra compacta (SNc) and the ventgral tegemental area (Smidt, Smits et al. 2004). It has been demonstrated that the aphakia (*ak)* mouse has no Pitx3 expression in the SNc and hence the DA neurons do not survive (van den Munckhof, Luk et al. 2003) thereby leading to aberrant behaviour and lower overall motor activity levels.

Three *PITX3* mutations have been reported till date in humans in AD cataracts and are listed in Table 1.2. Phenotypes include cataracts, anterior segment mesenchymal dysgenesis (ASMD) and neuro-developmental disorders.

1.13.3. Heat shock factor 4

Heat shock factor 4 (*HSF4*) is located on 16q22.1. *HSF4* has six transcripts- HSF4a to 4d, 4g, and 4h and three apparently aberrantly spliced mRNAs (Smaoui, Beltaief et al. 2004). *HSF4* belongs to the family of heat shock transcription factors that regulate the expression of heat shock proteins (HSPs) in response to different stresses, such as oxidants, heavy metals, elevated temperature, and bacterial and viral infections. *HSF4* has an amino-terminal helix-turn-helix DNA-binding domain which is the most conserved functional domain of HSFs. Trimerization of HSFs is mediated by the hydrophobic heptad repeats (HR-A/B) characteristic of helical coiled-coil structures (Sorger and Nelson 1989). Suppression of HSF trimerization is likely to be mediated by another region of hydrophobic heptad repeats (HR-C) that is present at the carboxyl- terminal of the protein. Because they lack HR-C, the HSF4 proteins are multimeric (Frejtag, Zhang et al. 2001; Pirkkala, Nykanen et al. 2001) Therefore, *HSF4* is constitutively a trimer that binds to HSE. In the human, HSF4 is widely expressed, especially in the heart, brain, skeletal muscle, lung, and pancreas (Nakai, Tanabe et al. 1997; Tanabe, Sasai et al. 1999). *HSF4* has at least two isoforms *HSF4a* and *HSF4b* (Tanabe, Sasai et al. 1999) and they are expressed in a tissue-specific manner (Nakai, Tanabe et al. 1997). *HSF4* regulates the genes for heat shock protein 70 (HSP70), HSP90, HSP27, and lens structural protein, αB-crystallin (Nakai, Tanabe et al. 1997; Somasundaram and Bhat 2004). In the mouse eye, expression of *HSF4* primarily in the form of HSF4b, which stimulates constitutive and inducible transcription of heat-shock genes, is high (Bu, Jin et al. 2002).

Recent observaions by Mou L and co-workers (2010) suggested that *HSF4* represses vimentin gene expression via the HSE-like element. The loss of *HSF4* function results in an increase in vimentin expression in *HSF4*^{tm1Xyk} knockout mice and affects lens differentiation, particularly impairing the denucleation of lens fiber cells (Mou, Xu *et al.* 2010).

Eight pathogenic mutations have been reported till date for autosomal dominant and recessive human hereditary cataracts (Table 1.2).

1.14. Membrane Proteins

1.14.1. Transmembrane protein 114

Transmembrane protein114 (*TMEM114*) is located on chromosome 16p31.3. The protein is predicted to contain 4 transmembrane domains with homology to lens intrinsic membrane protein (*LIM2*). In mice, Tmem114 is expressed in the eye, cerebellum and testis while in the eye the expression of Tmem114 is present particularly in the lens epithelial to fibre cell differentiation region. (Jamieson, Farrar et al. 2007). The expression of Tmem114 contrasts with that of beta-crystallin, which is expressed predominantly in the fiber cells, and not the epithelial cells. *In situ* hybridization at postnatal ages 2 and 6 weeks and adult ages showed similar patterns of expression (Jamieson, Farrar et al. 2007). Jamieson and co-workers have described a balanced translocation t(16;22)(p13.3;q22.1) associated with congenital/juvenile cataracts. The translocation breakpoint lies in the promoter region of *TMEM114* (Jamieson, Farrar et al. 2007) and disrupts transcription of the gene.

1.14.2. Major intrinsic protein

Major intrinsic protein (*MIP*) of the lens (*MIP26* or *AQP0*) is located on chromosome 12q14. The *MIP* of the vertebrate eye lens is the first identified member of a sequence-related

family of cell-membrane proteins that appears to have evolved by gene duplication (Shiels and Bassnett 1996). Predictions based on amino acid sequence suggest that members of the aquaporin family share a common topology which comprises of six transmembrane domains joined by five connecting loops (Francis, Chung et al. 2000). Several members of the *MIP* family transport water (aquaporins), glycerol and other small molecules in microbial, plant and animal cells. *MIP* is the most abundant junctional membrane protein (approx 50% of the total membrane protein) expressed in terminally differentiated fiber cells (Yancey, Koh et al. 1988) and is responsible for the maintenance of optical clarity in human lens (Berry, Francis et al. 2000). Besides its role as water channel (Geyer, Spence et al. 2006) it has been proposed to be a structural element as well as an adhesion molecule (Gruijters 1989; Lindsey Rose, Gourdie et al. 2006). The carboxy-terminal part of *MIP* proteins play role in lens development and transparency through its interaction with cytoskeletal protein filensin CP49, and connexin 45.6.(Yu, Yin et al. 2005; Lindsey Rose, Gourdie et al. 2006; Rose, Wang et al. 2008). Eight mutations have been described till date in MIP of which five are missense, two are deletion and one insertion. (listed in Table 1.2)

1.14.3. Gap junction / Connexin proteins

The connexin (Cx) gene family encodes gap junction proteins that form gap junction channels which allow the passage of ions (K^+,Ca^{2+}) and small molecules (<1kDa) including metabolites (e.g. glucose) and second messengers (IP3,cAMP and cGMP) between adjacent cells (Jiang and Goodenough 1996; Simon and Goodenough 1998; Donaldson, Kistler et al. 2001). In non-chordate animals a family of proteins called innexins form these channels while in chordates connexin proteins form hexamers known as connexons in cell membranes. A gap junction channel contains 12 subunit proteins (connexins) distributed among two

coaxially aligned hexameric hemichannels located in the plasma membranes of the opposing cells. Channels can cluster at appositional membranes and form gap junctional plaques (Saez, Berthoud et al. 2003). Gap junction channels are regulated through post-translational modifications of the carboxy-terminal cytoplasmic tail of the connexin proteins. Phosphorylation modulates assembly and physiological properties (Musil and Goodenough 1991; Lampe and Lau 2000; Dbouk, Mroue et al. 2009). Connexins are synthesized in the endoplasmic reticulum and are transported to the plasma membrane through the secretory pathway. Degradation of connexins involves the lysosomal and proteasomal pathways. The lysosomal pathway has been implicated in the degradation of gap junctions after their internalization. The proteasomal pathway has been implicated in the degradation of connexins as a quality control system for misfolded connexins and for connexins forming part of gap junctions (Berthoud, Minogue et al. 2003). The half-life (or turnover rate) of most connexins as determined by pulse-chase studies is 1.5-3hr (Laird 1996; Berthoud, Minogue et al. 2004). The human connexin genes are commonly divided into α, β, and an unnamed third group, all of which code for gap junction channels of different molecular weights (White, Srinivas et al. 2002). At least 20 connexin genes have been identified in humans with proteins ranging from 25 to 62 kDa (Evans and Martin 2002; Willecke, Eiberger et al. 2002). Mutations of specific connexin genes have been associated with several diseases including genetic deafness (Kelsell, Dunlop et al. 1997), skin disease (Richard, Smith et al. 1998), peripheral neuropathies (Bergoffen, Scherer et al. 1993), heart defects (Simon, McWhorter et al. 2004) and cataracts.

The lens expresses three distinct connexins CX43, CX46 and CX50, which are encoded by *GJA1* (chromosome 6q22.31), *GJA3* (chromosome 13q12.11) and *GJA8*

(chromosome 1q21.1) genes respectively (Kistler, Kirkland et al. 1985; Musil, Beyer et al. 1990; White, Bruzzone et al. 1992). All of these connexins belong to the α- connexin family and appear to have different functions in maintaining lens homeostasis (Gerido and White 2004). Cx43 is expressed mainly in the lens epithelial cells (Beyer, Kistler et al. 1989; Musil, Beyer et al. 1990); Cx46 and Cx50 are expressed in lens fibre cells (Paul, Ebihara et al. 1991; Goodenough 1992; White, Bruzzone et al. 1992). The loss of intracellular organelles in the differentiated lens fibre cells presents a unique homeostatic challenge, as these cells cannot support their own metabolism. Gap junctions contribute to lens function by coupling the metabolically active epithelium and the lens fibers into a syncytium (Goodenough 1979; Mathias, Rae et al. 1979; Mathias, Rae et al. 1997; Donaldson, Kistler et al. 2001). All connexins are predicted to have 4 transmembrane domains (M1-M4), two cytoplasmic (C1&C2) and two extracellular domains (E1&E2), amino- and carboxy-termini. 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 (Leube 1995). The pore-lining residues have been identified as lying in the first transmembrane domain and are essential for the formation of the pore, and therefore channel permeability (Kronengold, Trexler et al. 2003). Extracellular domains of connexins play an important role both in mediating hemichannel docking (Jiang and Goodenough 1996; Simon and Goodenough 1998) and regulation of voltage gating of the channel (Verselis, Ginter et al. 1994). White and co-workers have found that the specificity of heterotypic interactions between hemichannels composed of different connexins appears to be largely dictated by the primary sequence of the second extracellular loop (White, Bruzzone et al. 1994). Both the lens fiber cell connexins Cx46 and Cx50 form functional hemichannels when exogenously expressed in *Xenopus laevis* oocytes systems (Ebihara and Steiner 1993; Gupta,

Berthoud et al. 1994; Ebihara, Berthoud et al. 1995; Zampighi, Loo et al. 1999). Homozygous Cx43 knockout was lethal in mice but prenatal ocular development was found to be normal (White, Sellitto et al. 2001). Homozygous Cx46 knockout mice develop nuclear cataracts due to failure in maintenance of differentiation and of crystallin solubility while the homozygous Cx50 knockout mice show nuclear cataract with micropthalmia (Gong, Li et al. 1997; White, Goodenough et al. 1998). Targeted replacement of Cx50 with Cx46 by genetic knock-in corrected defects in cellular differentiation and presented cataract, but did not restore normal growth suggesting that intrinsic properties of Cx50 were required for cellular growth, whereas nonspecific restoration of communication by Cx46 maintained differentiation (White 2002). Although portions of the sequences of Cx50 and Cx46 are nearly identical, their hemichannel properties are quite different. Cx50 hemichannels are much more sensitive to extracellular acidification than Cx46 hemichannels and differ from Cx46 hemichannels both in steady-state and kinetic properties (Beahm and Hall 2002).

Gap junction protein 46 (Cx46) and Gap junction protein 50 (Cx50) are coded by the gene *GJA3* (chromosome13q12.11) and *GJA8* (chromosome 1q21.1) respectively. Cx46 is predominantly expressed in the lens (Mackay, Ionides et al. 1999) and functions in gap junction communications between elongated fibre cells (Paul, Ebihara et al. 1991). Fifteen mutations (fourteen missense and one frameshift mutation) have been reported in the literature till date in *GJA3/Cx46* protein in independent families, listed in Table 1.2 with AD hereditary cataracts. Twenty three mutations (twenty one missense and two frame shift mutations) have been linked to GJA8/CX50 gene with AD or AR modes of inheritance.

1.15. Miscellaneous genes:

1.15.1. Chromatin modifying protein - 4B

Chromatin modifying protein - 4B (*CHMP4B*) also known as charged multi vesicular body protein-4B (*CHMP4B*) is located on 20p12-20q12. It encodes a highly charged helical protein of approximately 25 kDa with N-terminal basic and C-terminal acidic halves. On the basis of phylogenetic analysis eleven CHMP genes have been identified in human genome and they have been divided into seven subfamilies, some with multiple members (Horii, Shibata et al. 2006; Hurley and Emr 2006). *CHMP4B* is one of three human orthologs of yeast Snf7/Vps32 (sucrose non fermenting-7 or vacuolar protein sorting-32), which functions in protein sorting and transport in the endosome-lysosome pathway. *CHMP4B* is a core subunit of the endosomal sorting complex required for transport–III (ESCRT-III), which facilitates the biogenesis of multivesicular bodies (MVBs) in simple yeast *Saccharomyces cerevisiae* (Hurley and Emr 2006). *CHMP4B* is found diffusely throughout the cytoplasm and/or in association with endosome–like compartments when expressed in cultured mammalian cells (Katoh, Shibata et al. 2003; von Schwedler, Stuchell et al. 2003). *CHMP4B* is thought to participate in the budding of a number of RNA viruses including the human immunodeficiency virus type-1 (HIV-1), from the surface of the infected cell (von Schwedler, Stuchell et al. 2003). Shiels A and co-workers had reported the first human mutation Asp129Val in a six-generation family from the United States with AD progressive childhood cataracts and Glu161Lys in a Japanese family with AD posterior polar cataracts in this gene (Shiels, Bennett et al. 2007).

1.15.2. Glucosaminyl (N-acetyl) Transferase 2 Gene

I/i antigens are carbohydrate moieties of glycoproteins and glycolipids on the cell surface of a variety of tissues and body fluids (Watanabe, Hakomori et al. 1979). The i antigen epitope is a linear poly-*N*-acetyllactosamine chain that has Gal-ß1 to 4GlcNAc-ß1-3 unit repeats, and the I antigen structure is branched by the addition of an *N*-acetylglucosaminyl (GlcNAc) residue through ß-1, 6 linkage to a galactosyl residue. Conversion of the i antigen into an I structure first takes place in human red blood cells during the first 18 months after birth as a result of the expression of a specific transferase, I-branching *GCNT2*. Glucosaminyl (*N*-acetyl) Transferase-2 gene (*GCNT2*) is located on 6p24.2. There are 19 variants from this gene as a result of alternative splicing. Three *GCNT2* splicing variants *GCNT2*A , *-B*, and *-C*, have identical exon 2 and 3 coding regions while they differ at exon 1 and are expressed differentially in specific tissues. While *GCNT2*A,-C code for 402 amino acids, *GCNT2B* codes for 403 amino acid residues. Mutation events that occur in the specific exon 1 region of the *GCNT2* gene may lead to a defect in one form of the *GCNT2* enzyme and i phenotype in certain cell types, whereas those that occur in the common exon 2 to 3 result in the i phenotype as well as congenital cataract, because of the elimination of activity of all three forms of the *GCNT2* enzymes (Inaba, Hiruma et al. 2003). Lack of this enzyme results in the adult i phenotype that is found to be highly associated with congenital cataract in Japanese (Ogata, Okubo et al. 1979) and among Chinese in Taiwan populations (Lin-Chu, Broadberry et al. 1991). Till date, three missense mutations have been reported in the literature (Table 1.2) with AR modes of inheritance.

1.15.3. Eph-receptor type-A2

Ephrin-receptor type-A2 precursor (*EPHA2* gene; chromosome 1p36.13) belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family. The ephrin receptors are divided into 2 groups based on the similarity of their extracellular domain sequences and their affinities for binding ephrin-A and ephrin-B ligands. The ephirin-A subclass comprises of 10 receptor genes (*EPHA1-10*) and the B subclass with 6 receptor genes (*EPHB1-6*). EPH and EPH-related receptors have been implicated in mediating developmental events, particularly in the nervous system (Herath, Spanevello et al. 2006). *EPHA2* gene encodes a protein that binds ephrin-A ligands (*EFNA1-6,EFNB1-3*) at cell contact sites to activate bidirectional signalling pathways effecting diverse physiologic processes including cell adhesion, repulsion, morphology and migration (Pasquale 2005; Pasquale 2008). Receptors in the EPH subfamily typically have a single highly conserved tyrosine kinase (SAM) domain and an extracellular region containing a cysteine-rich domain and 2 fibronectin type III repeats. This conserved ~70 amino acid SAM domain has a compact helical structure that is believed to facilitate protein–protein interactions. *EPHA2* is a type 1 membrane protein with extracellular NH_2-terminal and cytoplasmic COOH-terminal halves (Stapleton, Balan et al. 1999; Himanen and Nikolov 2003). Experiments on mice lacking ephrin-A5, a ligand of Epha2, develop cataracts as a result of impaired lens fiber cell adhesion (Cooper, Son et al. 2008).

Shiels and co-workers (Shiels, Bennett et al. 2008) first reported an *EPHA2* pathogenic mutation Gly948Trp resulting in dominant cataract in a four-generation American family of Western European origin. Zhang and co-workers had reported mutations in three families from different ancestral groups with dominant congenital cataracts. Of the three mutations reported Thr940Ile was identified in a Chinese family with posterior polar cataract,

one frameshift mutation (c.2915_2916delTG) with posterior polar cataract and a splicing mutation (c.2826-9 G>A) with total cataract were observed in a British family and Australian family respectively (Zhang, Hua et al. 2009). Kaul and co-workers, 2010 had recently reported a missense mutation resulting in an Ala785Thr in a consanguineous Pakistani family with AR cataract (Kaul, Riazuddin et al. 2010) (Table.1.2).

1.15.4. Galactokinase:

Galactokinase (*GALK1*) has been mapped to chromosomal location 17q25.1. The protein harbours two ATP binding sites (Stambolian, Ai et al. 1995). Galactokinase is involved in the first step of metabolism of galactose, the conversion of galactose to galactose -1-phosphate at the expense of ATP. Lack of the *GALK1* gene results in the accumulation of galactose which is further converted to galactitol by aldose reductase enzyme. Accumulation of galactitol and resulting accumulation of water results in cataracts (Hejtmancik and Kantorow 2004). Patients with homozygous galactokinase deficiency usually become symptomatic in the early infantile period and manifest cataracts (Stambolian 1988), galactoseemia, galactosuria, increased galactitol levels, and in few cases, mental retardation (Segal, Rutman et al. 1979). Although a cause and effect relationship between homozygous galactokinase deficiency and cataract is well established, the link between heterozygous galactokinase deficiency and cataract frequency remains uncertain (Skalka and Prchal 1980; Magnani, Cucchiarini et al. 1983; Stambolian, Scarpino-Myers et al. 1986).

Stambolian and co-workers had first identified two different mutations in unrelated families with galactokinase deficiency and cataracts (Stambolian, Ai et al. 1995). Yasmeen and colleagues have reported two mutations in separate Pakistani families with AR congenital

cataract (Yasmeen, Riazuddin et al. 2010). In one family, a single base pair deletion: c.410delG, which results in a frame shift leading to a premature termination of *GALK1*: p.G137fsX27. Additionally a missense mutation c.416T>C, resulting in a substitution of a Lue139Pro was also observed in a separate family by the same group.

1.16. Aim and scope of this study

This work deals with screening of candidate genes for pathogenic mutations in Indian patients with congenital hereditary cataracts of both AD and AR modes of inheritance. Limited studies were done on the genetics of hereditary cataracts in Indian population at the time of taking up this study. The approaches that can be used for study of cataract genes include mapping by linkage analysis and candidate gene screening. In our study we employed a candidate gene screening approach to screen for families with hereditary cataracts by single-strand conformation polymorphism (SSCP) followed by bi-directional sequencing. Since several families recruited were not suitable for mapping studies, a candidate gene approach was used. The cost-effectiveness combined with a fair sensitivity and specificity of the SSCP technique was the basis for using this technique. For this study, ten candidate genes that are major causes of congenital hereditary cataract were screened for mutations. Mutations that were novel and interesting were further characterized using transient expression in cell culture systems. The latter phase of the work presented in this thesis consists of experiments meant to determine the effects of novel mutations in the GJA8/Cx50 gene that were identified in this study on localization and stability of the mutant proteins. Mutations tested in the present study were of particular interest since the phenotypes resulting from these mutations were severe and two of the Cx50 mutations that were studied in vitro

were found to cause recessive cataract whereas the third mutation was found in a patient with dominant cataract.

1.16.1. Objectives

A. To identify mutations underlying hereditary congenital cataract in affected families.

B. To characterize the effects of cataract-causing mutations in Cx50 on the pattern of expression, localization and stability in relation to wild type Cx50 expression in suitable cell lines.

Chapter 2: Materials and Methods

2.1. Recruitment of patients and sample collection

The study protocol was approved by the institutional Review Board of L. V. Prasad Eye Institute, Hyderabad and adhered to the guidelines of the Declaration of Helsinki. Eligible patients (probands) with hereditary cataract either with a diagnosis of congenital or developmental cataract who presented to the Jasti V Ramanamma Children's Eye Care, L. V. Prasad Eye Institute, were recruited for the study. All first-degree relatives of the proband and, if there was history of disease in other relatives, all unaffected and affected members of those branches of the family that showed the disease were recruited. Clinical examinations were carried out for all the available members of the family, and were confirmed as far as possible by two independent ophthalmologists.

2.1.1. Inclusion Criteria

Bilateral lenticular opacity of congenital or developmental type. Two or more affected individuals in the family.

2.1.2. Exclusion Criteria

Probands who presented with any one of the signs mentioned below were excluded from the study:

a. Unilateral cataracts
b. Other ocular disease manifestations like glaucoma, micro-ophthalmos, anterior segment dysgenesis, uveal coloboma, vernal kerato-conjunctivitis (VKC), retinal degenerations etc.

c. Systemic disorders like microcephaly, cerebral palsy, mental retardation, Lowes syndrome, hyper-galactosemia, hyper-glycaemia etc.

d. Intrauterine infections (Toxoplasmosis, Rubella, Cytomegalovirus and Herpes simplex virus on the basis of history and examination of the child by a physician/pediatrician to rule out any systemic involvement)

e. Antenatal history of steroid use by the mother (steroid induced cataract)

f. Maternal history of steroid use.

g. Trauma

Patients who fulfilled the above criteria were explained the purpose of the study. Patients/families that were willing to participate were asked to sign a consent form, the contents of which were explained to them in their vernacular language. Family histories were taken and complete pedigrees were drawn. 4-6ml of blood was drawn in heparinised vacutainers (Greiner Bio-One, Kremsmuenster, Austria) by venipuncture from the arm vein. Blood samples were then stored at -20ºC.

2.1.3. Details of patients and families

A total of 40 families, which comprised of 184 individuals (100 effected and 84 unaffected) with autosomal recessive (AR) and with autosomal dominant (AD) forms of congenital hereditary cataracts were recruited in the study. Of the 40 families recruited 30 were presumed to be with AD and 10 families with AR modes of inheritance.

2.1.4. Controls

Blood samples from control individuals were collected after obtaining written informed consent. 100 control individuals were included in the study. All control individuals were without any history of congenital hereditary cataract.

2.2. Molecular genetic analysis

2.2.1. DNA Isolation

Isolation of genomic DNA from peripheral blood leukocytes was performed by a standard method involving phenol-chloroform extraction. Frozen blood samples were thawed at room temperature, lysis of red blood cells (RBC) was carried out by mixing with an equal volume of 1X phosphate buffered saline (PBS) followed by centrifugation at 4500 rpm to obtain a leukocyte pellet. The supernatant was discarded, and the pellet of white blood cells was treated with 35 µl of 20 mg/ml proteinase K (Bangalore Genei Ltd.**,** Bangalore, India) in 7.5 ml of buffer (0.01M Tris pH 8.0, 0.1M EDTA pH 8.0, 0.5% SDS), and 17.5 µl of 10 mg/ml RNase A (Bangalore Genei Ltd.**,** Bangalore, India) by incubating overnight at 37ºC. The lysate was extracted with equal volume of buffered phenol (equilibrated with 1M Tris pH 8.0 and maintained in 0.5M Tris pH 8.0). The upper aqueous layer was transferred to a fresh tube and mixed with an equal (1:1) volume of phenol-chloroform. This was followed by extraction with equal volume of chloroform. DNA from the aqueous phase was precipitated with 2.0M ammonium acetate (one-fifth volume of 10M stock solution) and 2 volumes of ethanol. The DNA was spooled on to a 1 ml pipette tip and washed with 70% ethanol. The DNA pellet was air-dried to remove residual ethanol. The DNA pellet was then dissolved in 500 µl of de-ionized water.

2.2.2. Quantitation of DNA

To estimate the concentration of DNA, 5 µl of the sample was transferred to a fresh 1.5 ml centrifuge tube and diluted to 1 ml with de-ionized water. Absorbance was measured at wavelengths 260 nm and 280 nm in a spectrophotometer (UV-1601, Schimadzu). An

optical density (OD) value of 1 corresponds to 50µg/ml for double-stranded DNA. The concentration of DNA was calculated using the given formula: Concentration (µg/ml) = OD (260) * 50* 200 (dilution factor).

2.2.3. Polymerase chain reaction

The primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3/) so as to generate amplified products of 350bp or less for single strand conformation polymorphism (SSCP) analysis. Primers were commercially obtained from Sigma-Aldrich, India. All the primers designed were 16 base-pairs or more in length with GC content in the range of 45-70%. Tm was calculated according to the formula Tm (ºC) = 2(A+T) + 4(G+C)-5. The annealing temperature for each pair of PCR primers was optimized experimentally. Polymerase chain reaction was performed using primers that were complementary to flanking intronic sequences of each exon. Primer sequences of primers used in this study and the PCR conditions for each pair are shown in Appendix 1, Table.2.1

PCR amplification was carried out with following reaction parameters

dNTPs	200µM
PCR reaction buffer	1X
Magnesium chloride	1.0 -2.5 mM
Primer (forward)	5-10 pmols
Primer (reverse)	5-10 pmols
Template DNA	50-100 ng
Taq DNA polymerase (Bangalore Genei Ltd., Bangalore, India)	1U

The final reaction volume was 25µl. Dimethyl sulfoxide (DMSO) was used at a concentration of 5% to 10% for GC-rich templates.

The cycling conditions used were as follows

1. Initial denaturation	94ºC, 2 min
2. Denaturation	94ºC, 45 sec
3. Annealing	50-70ºC, 30 sec
4. Elongation	72ºC, 45 sec
5. Final elongation	72ºC, 5-7 min
Cycles (steps 2-4)	35

2.2.4. Gel electrophoresis

2.2.4.1. Agarose gels

PCR products were checked by electrophoresis on 1.5% agarose gels to determine their amplification and quality. Agarose gels [1.5-2.0% (w/v) agarose; Seakem, Seakem Laboratories (India) Pvt. Ltd,.] were prepared by dissolving the required quantity of agarose in 1X Tris-acetate-EDTA (TAE; 0.04M tris-actete, 0.001M EDTA; pH 8) electrophoresis buffer by heating in a microwave oven, followed by an addition of 0.25µg/ml ethidium bromide (EtBr) from a stock solution of 5mg/ml. The agarose solution was poured into a gel tray containing a comb, allowed to cool and solidify as a gel. The gel was then placed in an electrophoresis tank and submerged in 1X TAE buffer. The DNA samples were mixed with DNA loading buffer (0.25% Bromophenol blue, 0.25% Xylene cyanol, 40% w/v Sucrose). Samples were loaded on the gel along with DNA size standard (MBI Fermentas, Lithuania). Horizontal electrophoresis was carried out at approximately 80-100V. DNA fragments were visualized on a UV transilluminator (UVI Tec, Cambridge, UK).

2.2.4.2. Polyacrylamide gels

PCR products digested with restriction enzymes were resolved on polyacrylamide gels. 8-10% gels were prepared from 30% acrylamide stock solution [(29:1; acrylamide: bisacyrlamide; Sigma-Aldrich, India] with 1X tris-borate EDTA (TBE) buffer (0.09M Tris-Borate, 0.002M EDTA; pH 8.3). Final volume was adjusted with autoclaved de-ionised water to 50 ml. Polymerization was done with the addition of 300µl of 10% ammonium per sulphate (APS) (Sigma-Aldrich, St.Loius, MO, USA) and 30µl of N,N',N',N-Tetramethylethylenediamine (TEMED) (USB, Cleveland, USA). The mix was then poured between glass plates (20*16cms) with 1.5 mm spacers. After polymerization samples were loaded in 6X loading dye (0.25% bromophenol blue, 0.025% xylene cyanol, 40% of w/v sucrose) and electrophoresis was carried out in a vertical electrophoretic unit (Hoefer SE 600 series, electrophoretic unit, Amersham Biosciences, San Francisco, USA.) at a voltage of 80-100V. The polyacrylamide gels were stained with EtBr (0.5µg/ml in 1X TBE) and DNA fragments were visualized on a UV transilluminator.

2.2.5. Single strand conformation polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) analysis is a mutation detection technique, which has a sensitivity range of 60% to 80% for fragments less than 300bp in size but decreases with increase in fragment size [Hayashi and Yandell, 1993]. The principle of SSCP is based on the conformation of single strands due to intra-strand base-pairing following denaturation and self-annealing. A single base substitution can potentially alter the conformation of the fragment and result in differential migration under conditions of non-denaturing electrophoresis. Therefore DNA samples having wild type (normal) and altered (variant) sequences display different mobility patterns.

For SSCP analysis, 2-3µl of PCR product was mixed with double the volume of 95% formamide containing bromophenol blue and xylene cyanol. Samples were denatured at 95ºC for 5 minutes and snap-chilled on ice. Samples were then separated on 6-10% polyacrylamide gel (19.5:0.5 acrylamide to bisacrylamide) containing 0.5X TBE and 5-10% glycerol. All samples were electrophorezed at room temperature and at 4ºC. Gels were run at constant voltage of 70V at room temperature and 120V at 4ºC. Gels were stained with 0.2 % silver nitrate for the detection of DNA. This was done by fixing the gels in 10% ethanol: 0.5% acetic acid for 45 minutes. Gels were then washed three times with de-ionized water followed by staining in 0.2% silver nitrate. They were then washed, developed in a solution of 1.5% sodium hydroxide and 0.4% formaldehyde until bands were visible at the desired intensity. Gels were washed in de-ionized water and photographed using UVI Doc gel documentation system over white light (UVI Tec, Cambridge, England). Fragments showing altered mobility relative to controls were sequenced directly.

2.2.6. PCR-RFLP

PCR-RFLP (Polymerase chain reaction–restriction fragment length polymorphism) analysis was used to screen for the presence of sequence variants in the control population as well as to ascertain segregation of sequence changes in the family members of probands. Sequence changes that led to the creation/abolition of recognition sites for restriction enzymes were tested by this method. Restriction enzyme sites were searched using the web-based program from New England BioLabs NEB cutter v.2.0 (http://tools.neb.com/NEBcutter2/index.php). PCR products were digested with appropriate units of the restriction enzymes, as mentioned by the manufacturer, in a final volume of 20-30µl using the recommended buffer. Reactions were incubated overnight or 3 hrs to 6 hrs at 37ºC or at the recommended temperature. 8-10%

polyacrylamide gel electrophoresis was used to resolve the restriction enzyme digested PCR products. The gels were then subjected to the EtBr staining and the separated DNA fragments were visualized on a UV transilluminator (UVI Tec, Cambridge, UK)

2.2.7. Gene sequences and nomenclature

Genomic or cDNA sequences of the genes screened for mutations were obtained from Ensembl database (http://www.ensembl.org) or Vega database (http://vega.sanger.ac.uk/index.html). The sequences obtained were compared with the publicly available sequence of the gene. Ensembl/Vega transcript IDs of the genes screened in this study are listed in Table 2.2. For nomenclature of sequence changes, numbering of residues was with reference to cDNA sequence and started with the first base of the initiation codon (ATG).

Table 2.2.

Gene Name	Ensembl /Vega Transcript ID
CRYAA	ENST00000291554
CRYBA1/A3	ENST00000225387
CRYBB2	OOTHUMT0000007501
CRYGB	ENST00000260988
CRYGC	ENST00000282141
CRYGD	ENST00000264376
GJA3	ENST00000241125
GJA8	ENST00000240986
HSF4	ENST00000264009
LIM2	OTTHUMT00000151603

Table 2.2: Transcript IDs of the gene sequences used as reference in mutation analysis.

2.3. DNA Sequencing

2.3.1. Purification of PCR Products

PCR products were purified to remove primers and dNTPs prior to sequencing by using Ultraclean PCR clean-up DNA purification kit (MOBIO laboratories, Carlsbad, California, USA) according to the protocol supplied by the manufacturer. The eluted DNA (50µl) in the collection buffer was precipitated by adding 2µl of 5M sodium chloride and 100µl of ethanol and centrifuged at 13000 rpm for 5 minutes. The DNA pellet was air dried and dissolved in 15µl of autoclaved de-ionised water. The PCR products were checked by agarose gel electrophoresis after column purification and subjected to sequencing.

2.3.2. Sequencing

PCR products were sequenced either commercially from MWG Ltd, Bangalore, India or by using Sanger's dideoxy chain termination method using fluorescently labelled ddNTPs, using the Big Dye version 3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA). Sequencing reactions were carried out in a 10µl reaction volume with 0.3- 0.5µl of Big Dye reaction mix, 3-5 pmols of primer and 50-100ng of amplified PCR product template. Cycle sequencing was done with initial denaturation at 96 ºC for 1 min, followed by 25 cycles of 96ºC for 10 sec, 50ºC for 5 sec, 60ºC for 4 min. The amplified products were purified by sodium acetate/ethanol precipitation. Briefly, PCR products were diluted to 100µl with de-ionised water followed by the addition of 3µl of 3M sodium acetate pH 4.6 (0.09M final concentration), and 250µl of ethanol. Samples were incubated on ice for 15 min followed by centrifugation at 12000 rpm for 20 min at room temperature. The pellets were then washed twice with 70% ethanol, air dried and dissolved in 10µl of de-ionized formamide

(Applied Biosystems Inc., Foster city, CA) and were analysed on the ABI 310 or 3130XL genetic analyzer.

2.4. Bioinformatics tools used

2.4.1. Multiple sequence alignment

Multiple sequence alignment of protein sequences was done to evaluate the degree of conservation of amino acid residues found to have mutations. Protein sequences were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov/sites/enterz). Homologous protein sequences were aligned using ClustalW2 software (http://www.simgene.com/ClustalW).

2.4.2. SIFT

The SIFT (Sorting Intolerant From Tolerant) tool (http://sift.jcvi.org/) was also used to predict the effect of sequence changes on the protein's function, based on homology search and the physical properties of amino acids. For calculating SIFT scores, the protein sequence of the candidate gene was downloaded from NCBI in the FASTA format. The SIFT scores range between 0 to 1 and SIFT scores <0.05 suggest that the amino acid change is not tolerated.

2.4.3. PolyPhen

PolyPhen (=*Poly*morphism *Phen*otyping) is a tool (http://genetics.bwh.harvard.edu/pph/) which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical

and comparative considerations. PolyPhen makes its predictions using three main sources of data:(1) FT, sequence annotation (or prediction) being a fragment of UniProt feature table (FT) describing the substitution position, (2) alignment, Position Specific Independent Counts (PSIC) profile scores derived from multiple alignment, (3) structure, structural information, obtained if a search against structural database was successful. The prediction is based on sequence annotation or sequence prediction or multiple alignment or structure. For calculating PolyPhen scores, the protein sequence of the candidate gene was downloaded from NCBI in the FASTA format. PolyPhen scores of >2.0 indicate the polymorphism is probably damaging to protein function; scores of 1.5–2.0 are possibly damaging; and scores of <1.5 are likely benign.

2.4.4. Human splice site finder (HSF)

HSF (Version 2.4.1) is a bioinformatics tool (http://www.umd.be/HSF/) that helps in predicting the effects of mutations on splicing signals or to identify splicing motifs if any in any human sequences. This software takes into consideration of all the available matrices (i.e. postion weight matrix, Maximal dependence composition, Markov model , Neural network, Feature generation algorithm etc.) for auxiliary sequence predictions as well as new ones developed by the Desmet and co-workers (Desmet, Hamroun et al. 2009). Only small rearrangements like substitutions, small exonic or intronic deletions and insertions, duplications and indels are best studied using this software. Individual values are generated and potential predictions are made based, whether there is any loss or creation of a cryptic splice site or branch site or enhancer motifs or silencer motifs due to the observed sequence change, based on well established predefined algorithms that would analyse the sequence

submitted to it in comparison with the reference sequence submitted or retrieved from various genome databases.

2.5. Generation of wild type and mutant Connexin 50 plasmid constructs

2.5.1. Preparation and transformation of competent DH5α.

Bacterial culture of DH5α was revived from a glycerol stock by streaking cells onto antibiotic-free LB agar plates to obtain isolated colonies. A single colony was picked and grown in LB medium overnight in the incubator at 37ºC. 1ml of overnight culture was added to 100 ml of fresh LB medium without antibiotic for preparing competent cells by the calcium chloride method as described by Nolan 1989. [Nolan, C., Ed. (1989).pp.1.82].

Transformations were performed with DNA (up to a volume of 5µl) and 100µl of competent DH5α cells in each tube. The cells with DNA was incubated on ice for 20 min, cells were heat-shocked in a water bath at 42ºC for 90 sec, and immediately placed on ice. 400µl of LB medium was added to the cells and they were then incubated at 37ºC for 45 min to allow for recovery of cells. A volume of 200µl of the culture was spread on LB agar plates with 50-100µg/ml ampicillin. The plates were incubated for 16 hrs in an incubator at 37ºC.

2.5.2. Isolation of plasmid DNA

Alkaline lysis method was used for the isolation of plasmid from bacterial cells either by following the method described in Sambrook *et al.,* (1989) or by using commercially available plasmid isolation kits from Qiagen (Plasmid Mini Kit QIAGEN, USA or Nucleobond PC 100 (Machery -Nagel GmbH & Co., Germany) following the instruction

manual. Cells from 1.5 ml of overnight culture were pelleted and cell pellets were re-suspended in 100µl of Glucose-Tris-EDTA (GTE) (solution I; Glucose 50mM, Tris pH 7.5, 25mM, EDTA 10mM), incubated on ice followed by lysis in 200µl of solution II containing 0.2N NaOH and 1% sodium dodecyl sulphate (SDS) and mixed by gentle inversion. The cell lysates were then treated with solution III containing 150µl of 5M potassium acetate (pH 4.8) on ice and then centrifuged at 13,000 rpm for 10 min. The supernatants were removed and DNA was precipitated with 800µl of ethanol. Isolated plasmid. DNA was then washed with 70% ethanol and air-dried. The pellet was re-suspended in 30µl of autoclaved de-ionized water containing 10µg/ml RNase A and allowed to incubate at 37ºC for 30 min. It was checked by electrophoresis on 0.8% agarose gel to check the quality with the 1kb DNA ladder (MBI, Fermentas).

2.5.3. Preparation of the wild type and mutant Connexin 50 plasmid constructs

2.5.3.1. Cloning Cx50 wild type into mammalian vector pcDNA3.1 (+)

The coding part of the GJA8/Cx50 gene was directly amplified using genomic DNA as template with *EcoR1* and *Xho1* sites incorporated within the forward and reverse primers, respectively (sequences of primers are given in Table 2.3). The wild type cDNA was then cloned into *EcoR1* and *Xho1* sites of the mammalian expression vector pcDNA3.1 (+).

Figure 2.1.

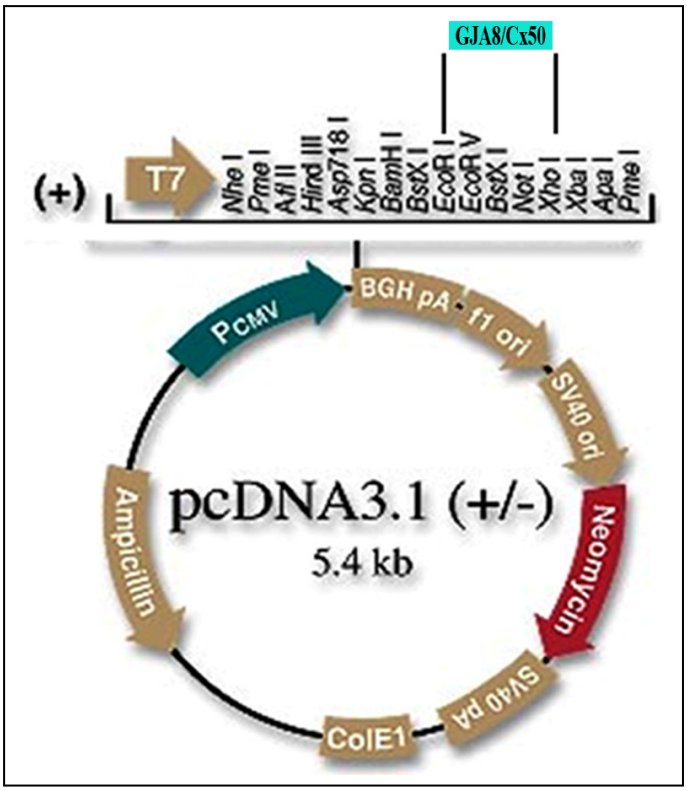


Figure 2.1. Map of pcDNA3.1 (+) vector along with the multiple cloning site and the location of insert

2.5.3.2. Generation of Cx50 mutants by the *Dpn1* method

Figure 2.2.

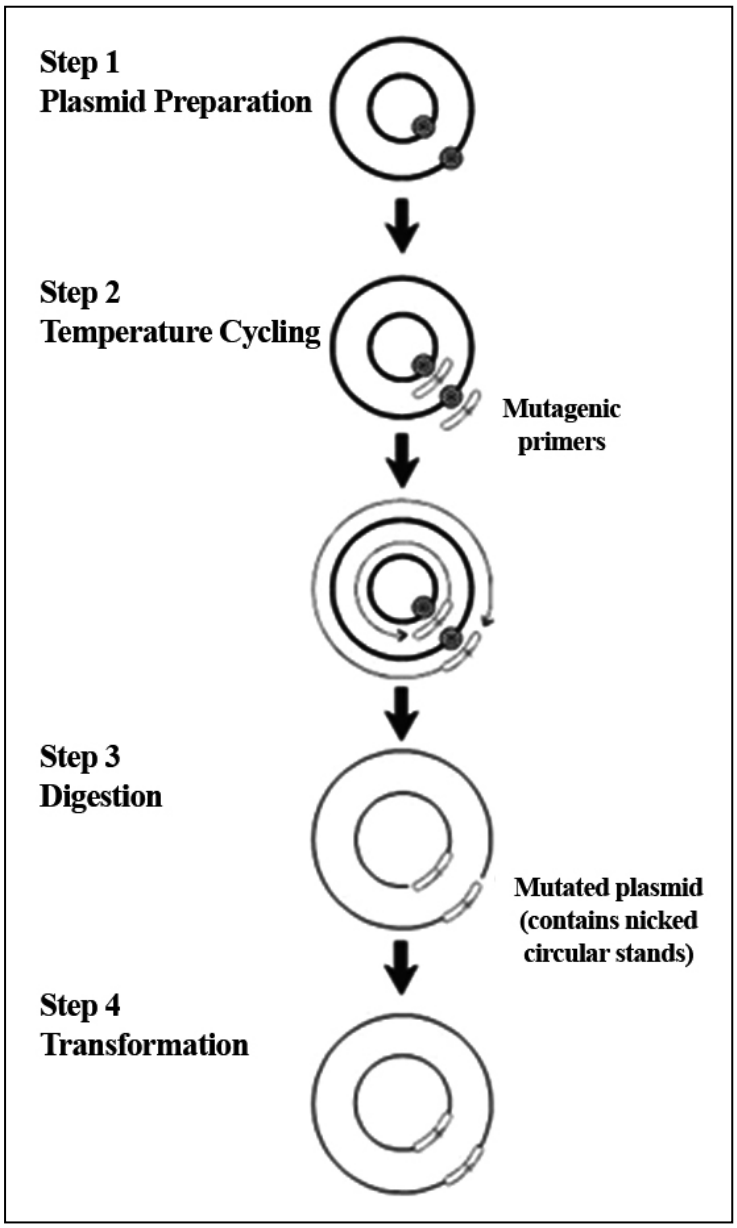


Figure 2.2: Schematic depiction of the *Dpn1* method of site-directed mutagenesis. Source: QuickChange ® Site-Directed Mutagenesis Kit manual p.7 (QIAGEN India Pvt. Ltd., India). The dark black line indicates the bacterial plasmid DNA. The grey line is the newly synthesised DNA.

Step 1: 50 ng of the pcDNA3.1 (+)-Cx50WT was used as template for PCR. The desired mutations were incorporated within the two primers (sequences of primers are given in Table 2.4).

Step 2: The divergent oligonucleotide primers, each complementary to opposite strands of the vector, were extended by PCR using a high fidelity *Pfu* DNA polymerase (MBI Fermentas, Lithuania). The cycling conditions used were as follows:

1. Initial denaturation	94ºC, 3 min
2. Denaturation	94ºC, 1 min
3. Annealing	52 ºC, 1 min
4. Elongation	68ºC, 14 min
5. Final elongation	68ºC, 1 hr
Cycles (steps 2-4)	16

Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks (shown in Figure 2.2).

Step 3: Following temperature cycling, 10 µl of the PCR product was treated with 1U/reaction of *Dpn1* enzyme (New England Biolabs, USA) for 1 hr at 37ºC. The *Dpn 1* endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *Escherichia coli* strains (dam^+) is methylated and therefore is susceptible to *Dpn 1* digestion.

Step 4: The nicked vector DNA containing the desired mutations was then transformed into 100µl of DH5α competent cells and then plated on LB-agar with ampicillin. The plates were incubated at 37ºC overnight. The colonies were inoculated into 2ml cultures, grown

overnight, and plasmid DNA isolated. The clones were then screened for the desired mutations by bi-directional sequencing using universal T7 forward and bovine growth hormone (BGH) gene transcription terminator reverse primers.

2.5.3.3. Subcloning of *GJA8* coding sequence into the pcDNA3.1/myc-His (-)A

The wild type cDNA was then subcloned into mammalian expression vector pcDNA3.1/myc-His (-) A, having the CMV promoter for expression and the myc and 6X-His epitopes at the carboxy-terminal end of the fusion protein, for detection using epitope-specific antibodies after transfection in mammalian cells. The coding region of the GJA8/Cx50 gene was amplified using hCx50-pcDNA 3.1(+) template and primers spanning the residues 1 (first base of the ATG codon) to 1275 of the GJA8/Cx50 cDNA. The forward primer was complementary to positions 1 to 21 of the cDNA with the *EcoR1* site incorporated in it (GJA8-MHF: 5'CCG**GAATTC**CAATGGGCGACTGGAGTTTCCTG'3) while the reverse primer was complementary to positions 1143 to 1275 with the *HindIII* site incorporated in it (MHR:5'CCC**AAGCTT**TACGGTTAGATCGTCTGACCTG'3). The restriction sites *EcoR1 and Hind III* are highlighted in bold in the sequences of forward and reverse primers.

2.5.3.4. Construction of the mutant clones in the pcDNA3.1/myc-His (-) A

Missense mutants of the GJA8 were generated in the wild type cDNA by *Dpn1* method as mentioned earlier in this chapter. Primers spanning the sites of the desired mutation, and designed to contain the mutant base at the appropriate position in the primer sequence, were used (sequences of primers are given Table 2.4). For creating the mutant cDNAs c.649G>A corresponding to the Val196Met , a mutant primer spanning positions 634 to 663 of the GJA8

cDNA was used with a single base substitution at the 16th position of the primer (shown in red in table 2.4, Appendix 1). For mutation c.658C>T corresponding to Pro199Ser, a mutant primer spanning positions 644 to 672 of the GJA8 cDNA was used with a single base substitution at the 15th position of the primer (Appendix 1, Table 2.4). For the double mutant c.649G>A+c.658C>T corresponding to Val196Met+Pro199Ser, a mutant primer spanning positions 633 to 672 was used with two single base substitutions at positions 16 and 25 in the primer (Table 2.4).

For the frameshift mutation coding for p.203Thr>AsnfsX47, *Dpn1* method was not employed. Instead, the pcDNA-hCx50 with an insertion of A at c.670insA construct, which was generated as described earlier in this chapter, was used as a template. The forward primer (GJA8-MHF: 5'CCG**GAATTC**CAATGGGCGACTGGAGTTTCCTG'3) was complementary to positions 1 to 21 of the GJA8/Cx50 cDNA with *EcoR1* restriction site while the reverse primer GJA8-26NRev (5'CCC**AAGCTT**GGAATCTCCCCCAGGGGC3') was complementary to positions cDNA positions 726 to 743 including the 3' end of the frameshifted cDNA and excluding the premature stop codon with the *Hind III* restriction site incorporated in it. The frameshift (insertion) mutation was at position 607 with respect to the first base of the ATG codon. The mutant frameshift sequence generates a stop codon and terminates at position 743. The PCR product so generated using the above-mentioned primers resulted in 763 bp fragment inclusive of forward and reverse primer sequences). This was then cloned into *EcoR1* and *HindIII* sites of the MCS of the -Myc-6X His A (-) vector such that the recombinant protein produced would have the myc and 6XHis epitope tags at the carboxy-terminus of the protein. The clones were then screened for the desired mutations by bi-directional sequencing using universal T7 forward and BGH reverse primers.

Figure 2.3.

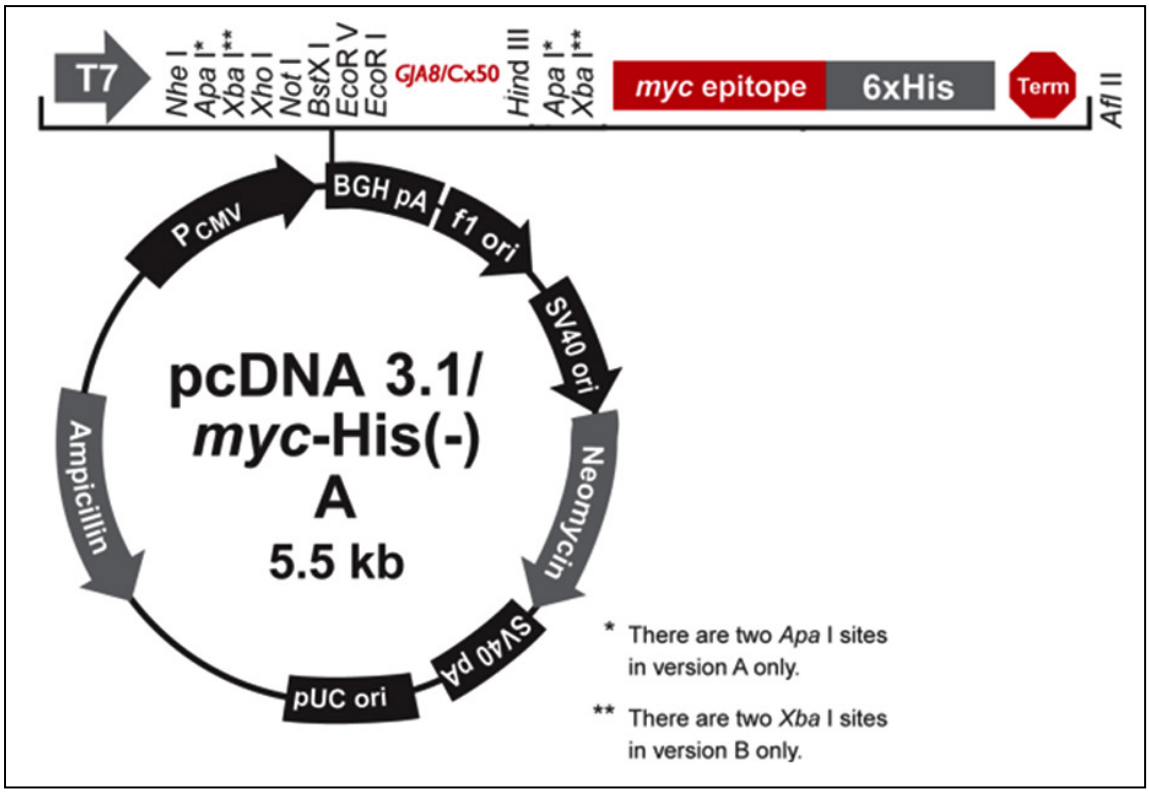


Figure 2.3: Vector map of the pcDNA3.1/myc-His (-) A with the GJA8/Cx50 insert cloned between EcoR1 & Hind III restriction sites.

Table 2.3.

Oligo Name	Sequence (5'-3')	Length (bps)
GJA8cDNA-F	GGGAATTCAGAAATGGGCGACTGGAGTTTC	30
GJA8cDNA-R	GGCCTCGAGTCATACGGTTAGATCGTC	27

Table 2.3: cDNA specific primers used for cloning the GJA8/Cx50 insert into pcDNA 3.1 (+). Red coloured nucleotide sequence in forward primer is *EcoR1* site. Green coloured nucleotide sequence in reverse primer is *Xho*1 site.

Table 2.4.

Oligo Name	Sequence (5'-3')	Length (bps)
Pro199Ser_F	GCTTCGTGTCCCGG**T**CCACGGAGAAAACC	29
Pro199Ser_R	GGTTTTCTCCGTGG**A**CCGGGACACGAAGC	29
c.670insA_F	GCCCACGGAG**A**AAAACCATCTTCATCCTG	29
c.670insA_R	CAGGATGAAGATGGTTTT**T**CTCCGTGGGC	29
Val196Met_F	GTGGTGGACTGCTTC**A**TGTCCCGGCCCACG	30
Val196Met_R	CGTGGGCCGGGACA**T**GAAGCAGTCCACCAC	30
Val196Met+Pro199Ser	GTGGTGGACTGCTTC**A**TGTCCCGG**T**CCACGGAGAAAAC	38
Val196Met+Pro199Ser	GTTTTCTCCGTGG**A**CCGGGACA**T**GAAGCAGTCCACCAC	38
BHGR	TAGAAGGCACAGTCGAGG	18
T7F	TAATACGACTCACTATAGGG	20

Table 2.4: Primers used in generation of theCx50 mutants by site directed mutagenesis by *Dpn1* method and screening for positive clones. The nucleotides highlighted in red colour are the desired sequence changes incorporated using commercially synthesized primers.

2.6. In-vivo Cx50 protein expression studies

2.6.1. Mammalian cell culture

HeLa cell lines were cultured on T25 culture flasks (Nunc, Rochester, NewYork, USA) with Dulbecco's modified Eagles medium (DMEM, Sigma-Aldrich, USA) and 10% fetal bovine serum (FBS, Sigma-Aldrich, USA). Cells were incubated at 37ºC with 5% CO_2. DMEM medium was prepared from 13.4g of DMEM powder in 1L of autoclaved de-ionized water, with addition of 3.7g sodium bicarbonate, 150 mg penicillin, 100 mg streptomycin and 2.5 mg Amphotericin B (Sigma-Aldrich, USA). The medium was sterilized by passing through 0.2 µm filter (Millipore, MA, USA). Cells were sub-cultured at 80% confluence by

trypsin treatment. Medium from the flask was removed and cells were washed with sterile 1X PBS (1X PBS ready to use powder was obtained from SIGMA-Aldrich Ltd, USA and was made up to 1L using de-ionized water and was autoclaved). 0.5 ml trypsin (0.125g, (0.125%) trypsin, 0.02 g (0.02%) EDTA in 100 ml of autoclaved de-ionized water) was added to cells, incubated for 2-3mins at 37ºC in the incubator and then trypsin was inactivated by addition of 1ml of complete medium (DMEM + 10% FBS). The cells were collected by repeated pipetting. Detached cells were seeded in a ratio of 1:3 to 1:6 onto fresh flasks containing 4 ml complete medium.

2.6.2. Cell culture and transfections for immunoflourescence (IF)

Hela cells were cultured on 18 mm^2 cover slips in a six-well culture dish with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), with necessary antibiotics at 37°C under humidified air containing 5% CO_2 for 16-18 hrs until 70-80% confluency is achieved. Transfections and immunofluorescence were carried out in the laboratory of Dr. Ghanshyam Swarup at CCMB, Hyderabad, with the help of Madhavi Muppirala. The protocol used for this was as developed and used in that laboratory. The cells were gently washed with serum-free medium twice prior to transfection. Transfections were carried out using lipofectamine-2000 transfection reagent (Invitrogen by life technologies, USA) in serum-free DMEM according to the protocol specified by the manufacturer. Amount of the plasmid DNA used was 300ng with 0.6µl of Lipofectamine reagent. Equal amounts of the hCx50-WT or mutant hCx50 plasmids (mutants p.203Thr>AsnfsX47, Val196Met, Pro199Ser and Val196Met+Pro199Ser) were transfected in Hela cells and expressed for 21hrs at 37ºC after which the cells were either treated with 30µg/ml of cycloheximide (Sigma-Aldrich, India) for 3 hrs or left untreated and then fixed. hCx50-WT or mutant hCx50

plasmids were also co-transfected with the plasmid encoding temperature-sensitive GFP-tagged vesicular stomatitis virus G-protein (VSVG-GFP ts045) described earlier by Presley et al 1997 (Presley, Cole et al. 1997). The VSVG-GFP construct codes for a VSVG-GFP fusion protein, which gets restricted within the endoplasmic reticulum (ER) when the cells are grown at 40ºC due to misfolding. At 32ºC the fusion protein translocates to the plasma membrane. Transfection mixes 1 and 2 containing 300 ng of plasmid DNA in 75 µl of DMEM and 0.6 µl of lipofectamine in 75 µl of DMEM, respectively, were pooled. The mix was incubated at room temperature for 30 min and added to the cells on cover slip drop by drop such that it covered the entire surface of the coverslip. The cells were the allowed to grow at 37ºC for 4 hours. The medium was then aspirated and replaced with 2 ml fresh medium and cells were allowed to grow at 40ºC until 21 hrs post transfection. The cells were then treated with cycloheximide at a concentration of 30µg/ml in DMEM (with serum and antibiotics) and shifted to 32ºC for 3 hrs. The cells were then fixed in 3.5% formaldehyde solution for about 10 minutes at room temperature and then washed with 1X PBS thrice and further processed for IF.

2.6.3. Immunofluorescent staining and confocal microscopy

The cells were washed once with PBS, fixed in 3.5% formaldehyde/ PBS permeabilized in 0.5% Triton X-100/PBS and 0.05% Tween-20/PBS and then blocked in 2% BSA and incubated with primary antibody for 2-3hrs at room temperature. Primary antibodies used were mouse monoclonal anti-c-myc antibody at a dilution of 1:500 (Sigma-Aldrich, USA) for detecting the myc epitope tag or anti-calnexin-rabbit polyclonal Ab (Santa Cruz Biotechnologies®, Inc., USA) at a dilution of 1:300 for detecting calnexin, which is a marker

for endoplasmic reticulum. This was followed by incubation with secondary Ab, Cy-3-conjugated anti-mouse or anti-rabbit IgG at a dilution of 1:1500 (Amersham Biosciences, USA) or anti-mouse AlexaFlour-488 (Molecular Probes, Invitrogen) for 1 hr each at room temperature. Counterstaining was done using DAPI (4', 6-diamidino-2-phenylindole). The fluorescent signals in transfected cells were observed and digital images were captured with the LSM 510 Meta NLO Confocal Microscope from Carl Zeiss (LSM 510, Carl Zeiss, Germany).

2.6.4. Cell culture and transfection for Western blot

Hela cells were cultured in standard 6-well plates at 50-70% confluence in DMEM supplemented with 10% FBS, and with necessary antibiotics at 37°C under humidified air containing 5% CO_2. The cells were transfected with pcDNA3.1 myc-His(-) A-hCx50 wild type and mutants using lipofectamine 2000 transfection reagent (Invitrogen, USA). 3µg of the plasmid DNA and 6µl of lipofectamine 2000 were used for transfection. 24 hrs post transfection the cells were washed once with 1xPBS and then 150µl of pre-warmed 1x Laemmli buffer was added to each well of the standard 6 well plate and then the cells were scraped using a cell scraper, boiled and stored at -80ºC.

2.6.4.1. SDS-PAGE

Discontinuous SDS polyacrylamide gel electrophoresis (gel size, height x width, 10 x 10) was performed with separating and stacking gels of 10% and 5% acrylamide respectively on a vertical electrophoresis system (Hoefer**,** AP Biotech UK Ltd, Buckinghamshire, England). 15-20µl of cell lysate was loaded and electrophoresed under standard conditions.

Separating and stacking gels were made up as shown below

Resolving gel:

De-ionized water	- 4.54 ml
30% (29:1) acrylamide	- 2.7 ml (10% final)
5M (4X) Tris pH 8.8	- 2.5 ml (1X final)
10% SDS	- 100µl (0.1% final)
10% APS	- 100µl (0.1% final)
TEMED	- 6µl
	10.00 ml

Stacking gel:

De-ionized water	-1.72 ml
30% (29:1) acrylamide	- 0.50 ml (5% final)
0.5M (4X) Tris pH 6.8	- 0.76 ml (1X final)
10% SDS	- 30µl (0.1% final)
10% APS	- 30µl (0.1% final)
TEMED	- 3µl
	3.060 ml

20µl of cell lysate (corresponding to about 1/8th of a 6-well culture dish) was loaded on the gel and electrophoresis was performed with 1X Tris-glycine SDS buffer (0.25 M Tris, 19.2 mM glycine, 0.01% SDS, pH 8.3) at a constant voltage (100V) for 2 hours or more. After electrophoresis the gel was kept in transfer buffer (1XTris-glycine-SDS with 20% methanol) for 15 min. The proteins were transferred on to polyvinylidene fluoride (PVDF) membrane by using a semi-dry transfer unit (TE70, GE Healthcare UK Ltd, Buckinghamshire, England).

2.6.4.2. Western blot

PVDF membrane and 6 filter papers (Whatman # 3) were cut to the same size as the gel and soaked in 1X transfer buffer (0.25 M Tris, 19.2 mM glycine and 0.01% SDS with 20% methanol) for 15 min. The transfer set-up was made by placing from bottom to top, 3 filter papers, PVDF membrane, gel and 3 filter papers on one another, in the same order on the lower lid of the apparatus. Air bubbles were removed by rolling a glass tube on top of the transfer setup. The upper lid was placed and transfer was done with a constant voltage of 15V for 1 hr.

Immunodetection of Cx50/GJA8 protein was carried out by first placing the membrane in blocking buffer (1XPBS, 5% non-fat milk and 0.05% Tween-20) for 2 hrs at room temperature, followed by incubation with primary antibody (either monoclonal mouse anti-c-myc Ab (Sigma-Aldrich, USA) at a dilution of 1:3000 or goat polyclonal anti-connexion-50 (Santa Cruz, USA) at a dilution of 1:3000 for 1 hr at room temperature. The membrane was then washed 3 times with PBS (containing 0.05% Tween-20) and incubated with secondary antibody (anti-mouse IgG conjugated with horseradish peroxidise (HRP) (Sigma-Aldrich, USA) or donkey anti-goat HRP conjugated (Abcam, UK) at dilutions of 1:3000 for 1 hr at room temperature. The membrane was washed three times with PBS-Tween 20 (0.05%). Detection was done by enhanced chemiluminescence method. One ml of solution 1 (0.45 mM Coumaric acid, 2.5 mM luminol, 100 mM Tris pH 8.5) was mixed with 1 ml of solution 2 (5 mM H_2O_2, 100 mM Tris, pH 8.5) before pouring on to the membrane. Signal was detected by exposing the blot to an X-Ray film in an autoradiography cassette, followed by development of the film.

Chapter 3: Mutational screening of candidate genes

This chapter describes the screening of candidate genes for hereditary congenital cataract in 40 families with autosomal recessive (10 families) and dominant (30 families) inheritance, Ten genes -*CRYAA, CRYBA3/A1, CRYBB2, CRYGB, CRYGC, CRYGD, GJA3, GJA8, HSF4* and *LIM2*, were screened for sequence changes.

3.1. Pathogenic sequence changes observed

Of the gene mutations that are reported so far in congenital cataracts, the majority of mutations occur in the family of crystallin genes [reviewed by (Graw 2004; Hejtmancik 2008; Graw 2009)]. Mutations in the 2 connexin genes (GJA3 and GJA8) are also frequent causes of congenital cataract followed by smaller numbers of mutations in the heat shock transcription factor-4 (*HSF4*), aquaporin-0 (*LIM2, AQP0, MIP*), and beaded filament structural protein-2 (*BFSP2*) genes (Devi, Yao et al. 2008; Sajjad, Goebel et al. 2008). Of ten genes that were selected in the present study, six genes code for crystallin proteins (*CRYAA, CRYBA3/A1, CRYBB2, CRYGB, CRYGC, CRYGD*), two genes encode lens gap junction proteins (*GJA3* and *GJA8* for connexins 46 and 50 proteins respectively), one gene encodes heat shock transcription factor (*HSF4*) and one gene encodes the membrane protein *LIM2* (listed in appendix Table 1.2). All the exons and the flanking intronic regions of the genes selected were PCR-amplified and the products were subjected to single strand conformation polymorphism (SSCP) analysis. The variants on SSCP analysis were subjected to bidirectional sequencing by automated methods. Screening for the observed mutations was performed on ethnically matched unrelated

normal controls by either SSCP/Restriction fraction length polymorphism (RFLP). Details of the protocol for SSCP/RFLP are in Chapter 2.

Genetic analysis revealed 6 sequence changes in the *GJA8, GJA3* and *LIM2* genes in 6 families that were presumed pathogenic mutations. Three of these changes were homozygous and were found in patients with autosomal recessive disease, and three changes were heterozygous, found in patients with autosomal dominant cataract. Homozygous changes found were a homozygous insertion A at codon 203 (c.670insA p.203Thr>AsnFsX47), in GJA8/Cx50, a missense change of c.649G>A (Val196Met) in GJA8, and a missense change c.587 G>A (Gly154Glu) in *LIM2/MP19*. Heterozygous changes found were c.658C>T (Pro199Ser) in GJA8/Cx50, c.589C>T (Pro197Ser) and c.84G>A (Val28Val) in GJA3/Cx46. The 6 mutations mentioned co-segregated with the disease phenotype in each of the families and were absent in at least 50 ethnically matched unrelated normal controls.

3.1.1. Family 26: c.670insA p.203Thr>AsnfsX47 in the *GJA8* gene

Figure 3.1A

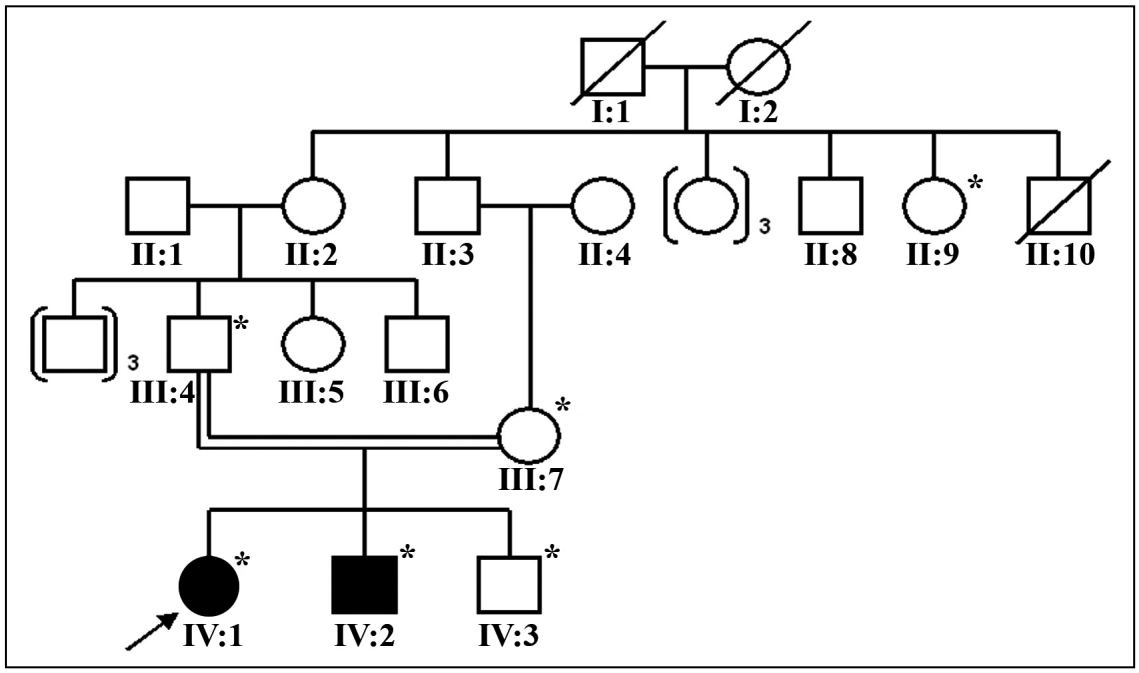


Figure 3.1A: Pedigree of family 26 with autosomal recessive cataract. The dark symbols indicate affected individuals and open symbols indicate unaffected individuals. Symbols marked with asterisks represent individuals who were genetically analyzed

Table 3.1.

No.	Patient	Age at presentation (years)	Genotype	Age of onset	Clinical Status
IV:1	Proband	12	Homozygous insertion	Since birth	Aphakia (OU), nystagmus & dense amblyopia
IV:2	Brother	10	Homozygous insertion	Since birth	Total cataract (OU), nystagmus & dense amblyopia
IV:3	Brother	8	Heterozygous insertion	Normal	Normal
III:4	Father	37	Heterozygous insertion	-	simple myopia (OD)
III:7	Mother	30	Heterozygous insertion	-	Normal vision. Early cataract (OU)
II:9	Maternal grand aunt	42	Normal	Normal	Normal

Table 3.1: Mutational and clinical status of individuals from family 26

In family # 26 (Figure 3.1A) with AR cataract, a single base homozygous insertion of A at codon 203 (corresponding to c.670, located in exon 2) was observed in *GJA8* in two affected individuals (IV:1, IV:2) whereas the other unaffected individuals (III:4, III:7, II:9) were heterozygous for the same (Figure 3.1B). The homozygous insertion that was found in codon 203, predicted to be in the 2nd extracellular domain of connexin 50. This insertion is predicted to result in a frameshift at this position followed by termination

resulting in a truncated protein of 248 residues (Figure 3.1C). Screening of 75 ethnically matched normal controls for the c.670insA change was done by SSCP. None of the screened controls had the pathogenic change observed in this family. Listed below are the representative sequence electropherograms of individuals from family 26.

Figure 3.1B.

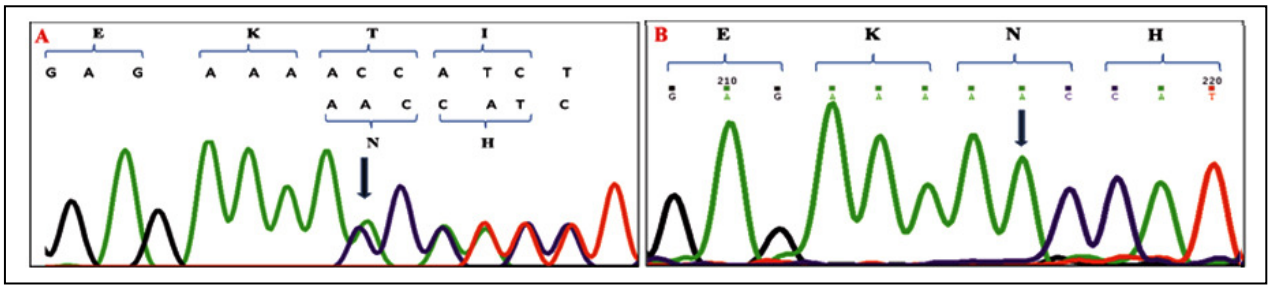


Figure 3.1B: Sequence of the *GJA8* coding region. The sequence of *GJA8* showing the insertion (arrow) of an A at position 670 of the cDNA (c.670insA) homozygous in the proband (panel B) and heterozygous in unaffected parent (panel A). Codons are marked by brackets and amino acids are indicated above. Codons and their corresponding amino acids for both wild type and mutant alleles are shown in panel A while the altered reading frame, due to insertion of A, are shown in Panel B

Figure 3.1C.

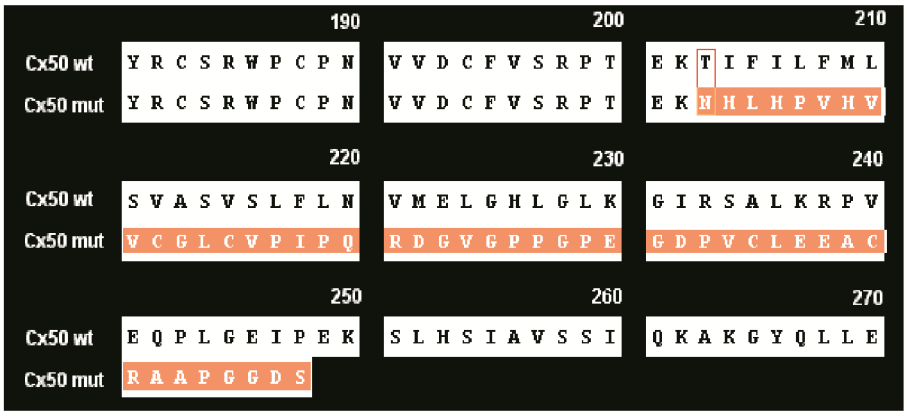


Figure 3.1C: Sequences of wild type and mutant GJA8 proteins, Partial protein sequences of the wild type GJA8/Cx50 (wt) and predicted sequence of the insertion mutant (mut) c.670 ins A, p.Thr203AsnfsX47 are shown. The residue (position 203) at the start of the frameshift is boxed. The mutant protein terminates at 248 amino acids. Residues are numbered with respect to the wild-type Cx50 sequence.

3.1.2. Family 32: c.649G>A (Val196Met) in *GJA8* gene

Figure 3.2A

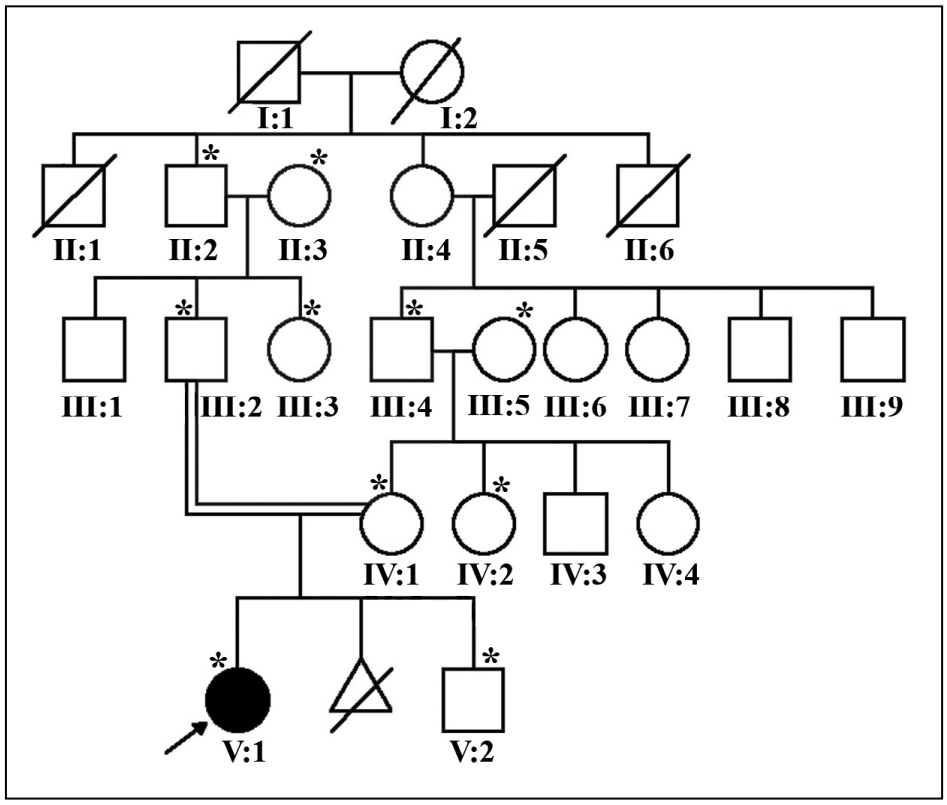


Figure 3.2A: Pedigree of family 32 with autosomal recessive cataract. Symbols marked with asterisks represent individuals who were genetically tested.

Figure 3.2B.

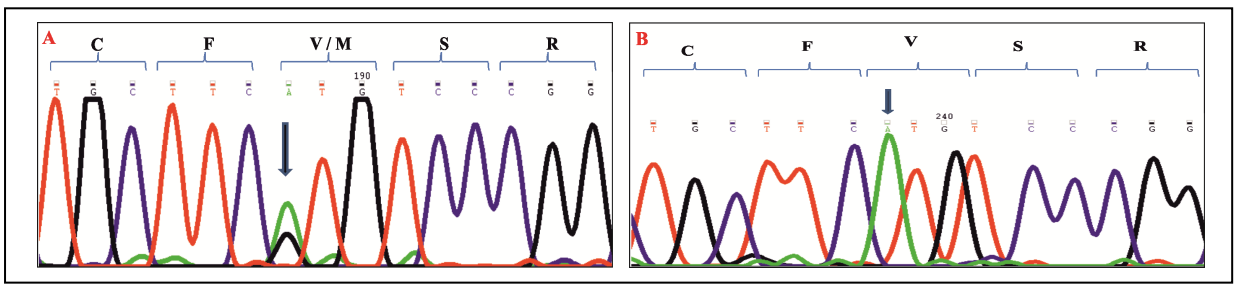


Figure 3.2B: The sequence of GJA8 showing homozygous substitution of G>A in the proband (Panel B) where as the unaffected mother was heterozygous for the same. (Panel A). Arrow shows the site of mutation and the codons are marked by brackets.

In family 32 (Figure 3.2A) with autosomal recessive cataract, the proband (V: 1) was found to carry a homozygous substitution of c.649G>A resulting in Val196Met in *GJA8*/CX50. The parents (III:2 and IV:1), sibling (V:2), and other relatives (IV:V, III:4, II:2) were found to be heterozygous, whereas individuals (II:3, III:3, III:5) were found to carry 2 copies of the wild type allele. The observed change is predicted to be in the second extracellular domain of the GJA8/Cx50 protein. Screening of 75 ethically matched controls by SSCP showed this change to be absent. Shown below are the representative electropherograms of individuals from family 32 (Fig 3.2B).

Table 3.2.

S.No.	Patient	Age at presentation (yrs)	Genetic status	Clinical status
V:1	Proband	1.8	Homozygous GTG>ATG	Congenital cataract (OU)
V:2	Sibling	2 months	Heterozygous GTG>ATG	Normal
IV:1	Mother	25	Heterozygous GTG>ATG	Dot-like opacities in lens (Developmental cataract) unaided VA : 6/6 & N6 (OU)
IV:4	Maternal aunt	17	Heterozygous GTG>ATG	Normal
III:2	Father	30	Heterozygous GTG>ATG	Simple myopia (OU) unaided VA : 6/6 & N6(OU)
III:3	Paternal aunt	28	Homozygous GTG	Normal
III:4	Maternal GF	52	Heterozygous GTG>ATG	Refractive error
III:5	Maternal GM	45	Homozygous GTG	Refractive error
II:3	Paternal GM	52	Homozygous GTG	Refractive error
II:2	Paternal GF	58	Heterozygous GTG>ATG	Refractive error

Table 3.2: Mutational and clinical status of individuals from family with the *GJA8* mutation in family 32

3.1.3. Family 45: c.587 G>A (Gly154Glu) in *LIM2*

Figure 3.3A:

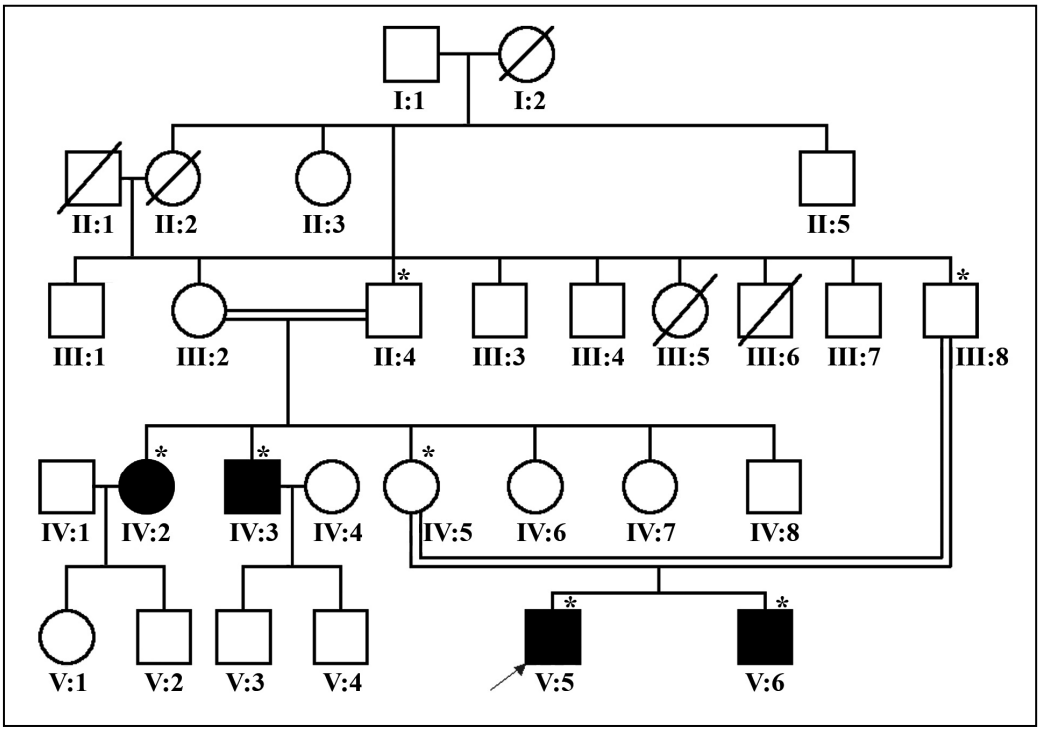


Figure 3.3A: Pedigree of family 45 with autosomal recessive cataract. Symbols marked with asterisks show individuals screened for mutations.

Figure 3.3B.

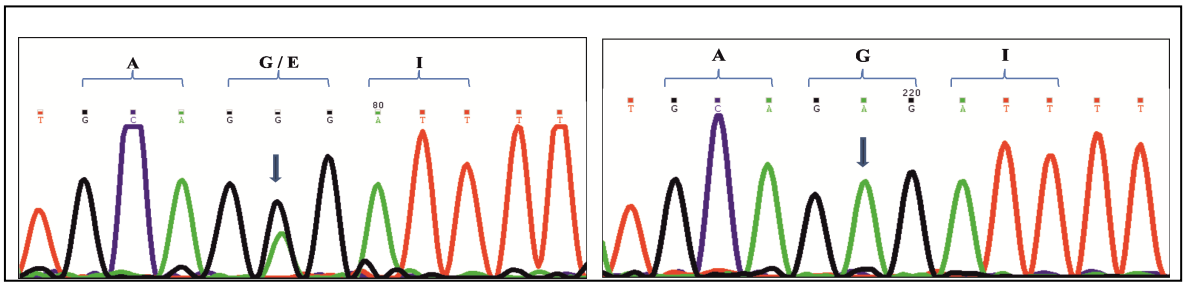


Figure 3.3B. Sequence electropherogram showing mutation in *LIM2*. Heterozygous c.587 GGG>GAG change found in unaffected members (Panel A) of the family and homozygous GGG>GAG resulting in Gly154Glu at this position in affected individuals (Panel B). Arrow indicates site of mutation.

Screening of the *LIM2* gene showed a substitution c.587G>A in exon 5 predicting an amino acid change of Gly154Glu in family 45 (Fig.3.3A) with autosomal recessive cataract. There were four affected individuals from two generations. All 4 affected individuals of the family (IV:2, IV:3,V:5,V:6 in Figure 3.3A) were homozygous for this change and unaffected parents II:4, II:8 & IV:5 were heterozygous. Screening of 75 unrelated normal controls for this change by SSCP showed it to be absent.

Table. 3.3.

S.No.	Patient	Age at presentation (yrs)	Age of onset	Genetic status	Clinical status
V:5	Proband	6	3	GGG>GAG (Homozygous)	Total cataract, surgery at 3ys, amblyopia
V:6	Sibling	4	1.5	GGG>GAG (Homozygous)	Total cataract, surgery at 3ys, amblyopia
IV:2	Maternal aunt	35	Since birth	GGG>GAG (Homozygous)	Surgery at 3, nystagmus, amblyopia
IV:3	Maternal uncle	29	<8	GGG>GAG (Homozygous)	Surgery at 10, nystagmus, amblyopia
IV:5	Mother	27	WNL	GGG>GAG (Heterozygous)	Normal
III:8	Father	35	WNL	GGG>GAG (Heterozygous)	Normal
II:4	Maternal grandfather	55	WNL	GGG>GAG (Heterozygous)	Normal

Table. 3.3 Mutational and clinical status of individuals from Family 45.

WNL' refers to within normal limits

3.1.4. Family 21: c.658C>T Pro199Ser in the *GJA8* gene

Figure 3.4A:

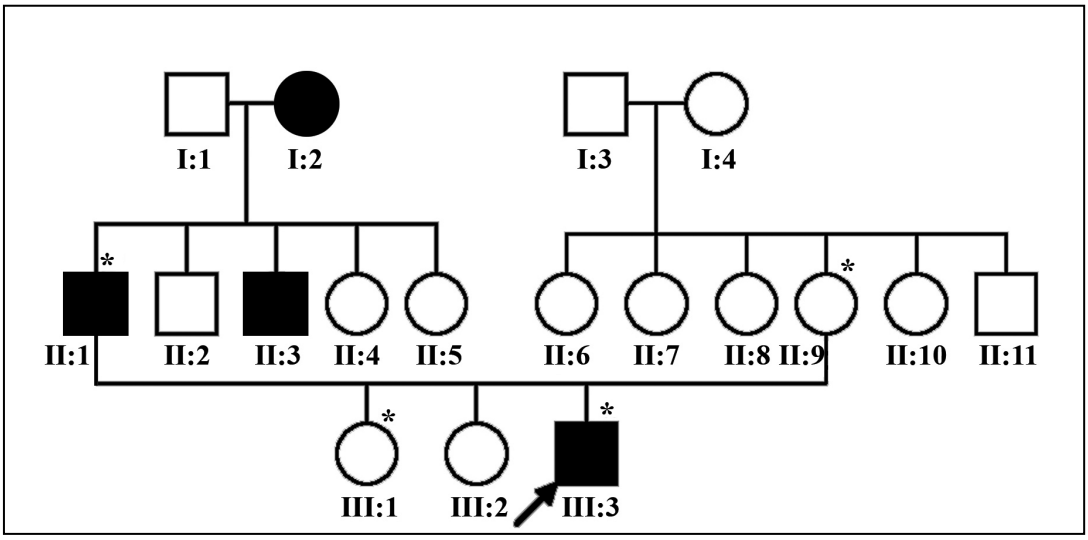


Figure 3.4A: Pedigree of family 21 with autosomal dominant cataract. Symbols marked with asterisks represent individuals who were genetically & clinically tested.

Figure 3.4B.

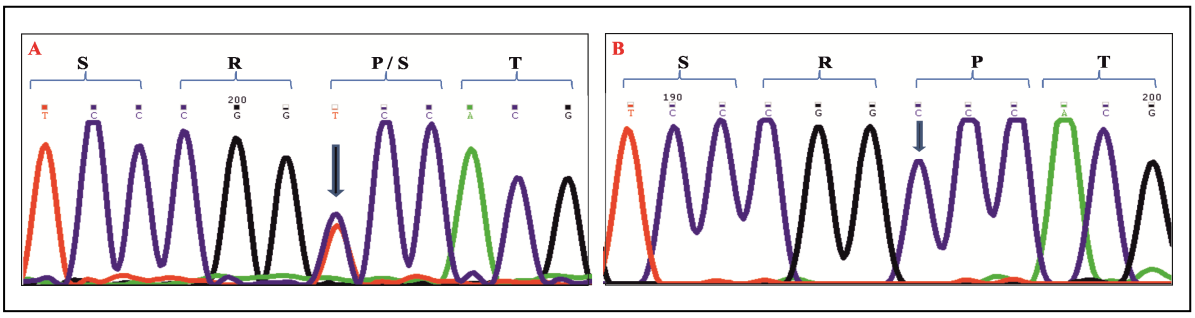


Figure 3.4B. The sequence of GJA8 showing the heterozygous substitution of CCC>TCC (Pro>Ser) at c.DNA residue 658 in the proband (Panel A) whereas the unaffected mother had the wild type allele (Panel B). The site of mutation is indicated by an arrow.

In family 21 (Figure 3.4A), with autosomal dominant cataract, a heterozygous substitution of c.658 C>T resulting in Pro199Ser in GJA8/Cx50 was observed in diseased

individuals (II:1, III:3) whereas the individuals III:1 and II:9 had 2 copies of the wild type allele.

Table 3.4.

S.No.	Patient	Age at presentation (yrs)	Genetic status	Age of onset	Clinical Status
III:1	Sibling	7	CCC (Normal)	-	Normal
III:3	Proband	7 months	Heterozygous CCC>TCC	3 Months	Total cataract (OU)
II:1	Father	34	Heterozygous CCC>TCC	Since birth	Aphakia (OU)
II:9	Mother	25	Homozygous CCC	-	Normal

Table 3.4: Mutational and clinical status of individuals from family 21

The Pro199Ser change is predicted to be in the second extracellular domain of the GJA8/Cx50 protein. The observed change resulted in creation of a recognition site for the enzymes *HaeIII* and *BsuR1*. 75 ethnically matched normal controls were tested for this change by PCR-RFLP analysis and it was found to be absent. Listed below are representative electropherograms of the individuals from family 21 (Figure 3.4B).

3.1.5. Family 41: c.589 C>T Pro197Ser in the *GJA3* gene

Figure 3.5A.

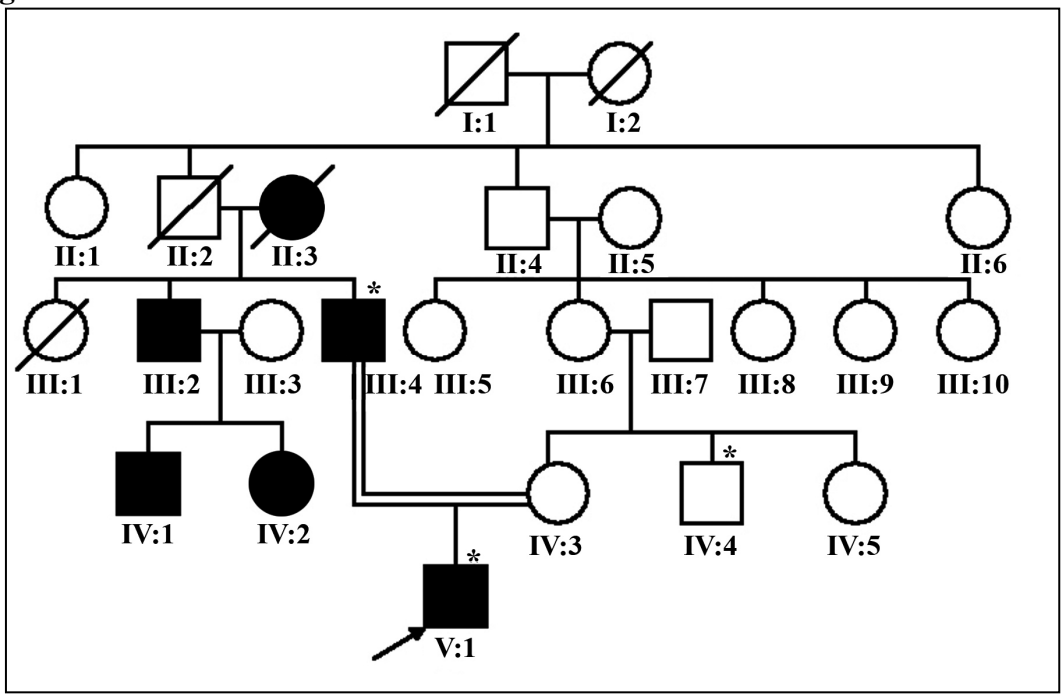


Figure 3.5A: Pedigree of family 41 with autosomal dominant cataract.

Figure 3.5B.

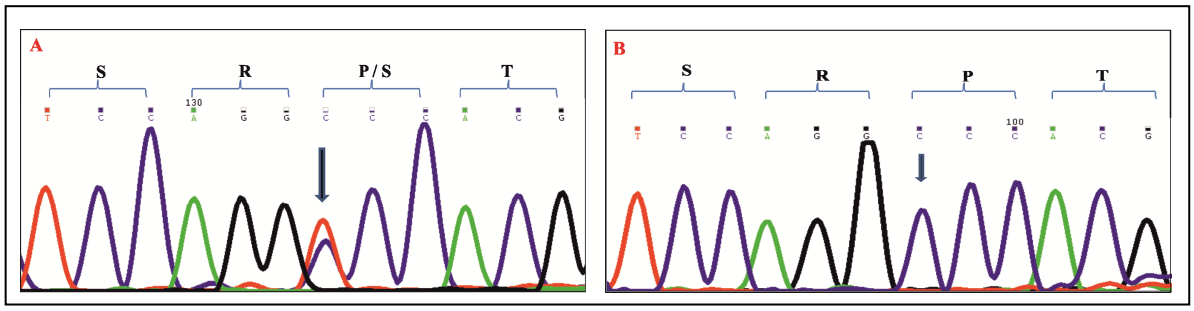


Figure 3.5B: Sequence electropherogram showing mutation in *GJA3*. Heterozygous c.589 C>T change (arrow) involving CCC>TCC (Pro197Ser) found in affected members of the family. Mutation is shown in the panel A whereas the panel B shows wild type allele C (arrow at the site of mutation).

In family 41 (Figure 3.5A), a heterozygous substitution c.589C>T resulting in Pro197Ser in GJA3/connexin 46 was observed in 2 affected individuals (III:4 and V:1) whereas an

unaffected individual who was maternal uncle of the proband (IV: 4) had the wild type alleles. The observed change is predicted to be in the second extracellular domain of the GJA3/Cx46 protein. This change resulted in creation of a recognition site for the *AvaII* enzyme. PCR-RFLP analysis revealed that this change was absent in 75 ethnically matched normal controls. Listed below are the representative electropherograms of individuals of family 41 screened by automated sequencing (Figure 3.5B).

Table 3.5.

S.No.	Patient	Age at presentation (yrs)	Age of onset	Genetic status	Clinical status
V:1	Proband	11	First decade	Heterozygous CCC>TCC	Developmental lamellar cataract with riders (OU)
IV:4	Maternal uncle	28	Normal	Homozygous CCC	Normal
III:4	Father	37	Not known	Heterozygous CCC>TCC	Operated for cataract in both eyes at age 30 years; posterior capsular opacification in right eye

Table 3.5: Mutational and clinical status of individuals from family with the *GJA3* mutation in family 41

3.1.6 Family 39: c.84 G>A Val28Val in the *GJA3* gene

Figure3.6A.

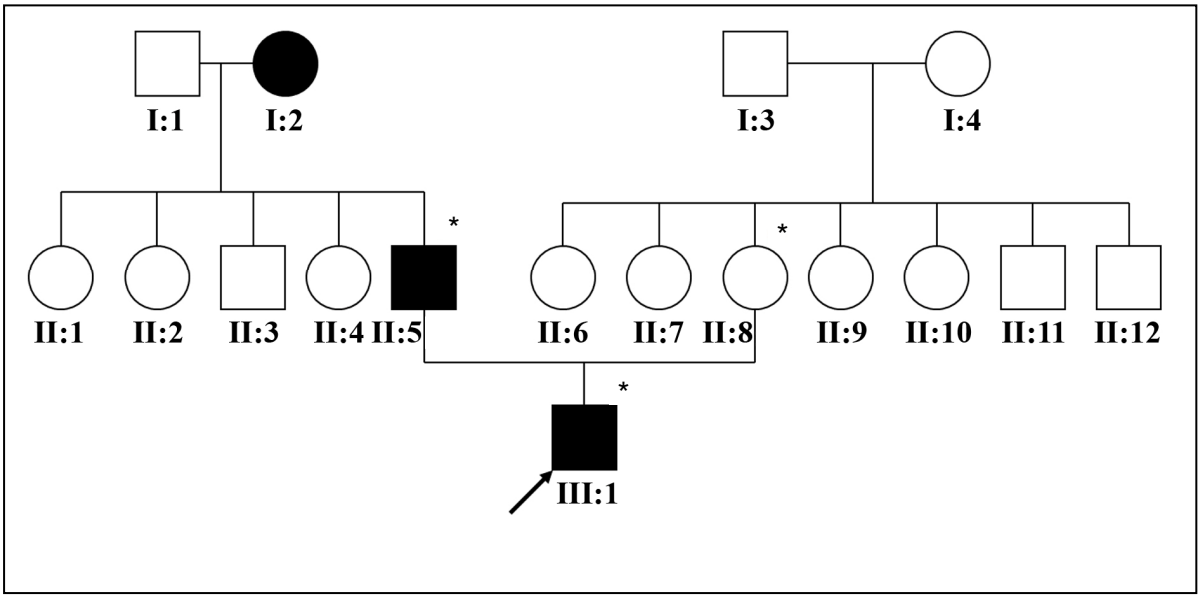


Figure 3.6A: Pedigree of family 21 with autosomal dominant cataract

Figure 3.6B.

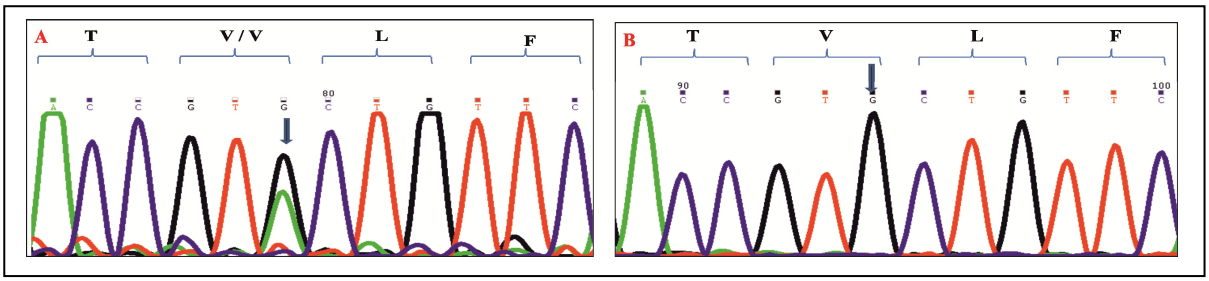


Figure 3.6B. Sequence electropherogram showing a silent mutation in *GJA3*. Heterozygous c.84GTG>GTA change found in affected members (Panel A) of the family and normal sequence in unaffected individuals (Panel B). Arrow indicates site of mutation.

In family 39 (Figure 3.6A), a heterozygous substitution of c.84G>A in GJA3/connexin 46 resulting in a silent mutation Val28Val was observed in affected individuals (III: 1, II: 5) whereas the individual (II: 8) had the wild type allele for the same. The observed change is predicted to be in the first transmembrane domain of the *GJA3*/Cx46 protein (Devi, Reena et al. 2005). This change was found to be segregating with the disease phenotype and screening of 50 unrelated normal controls for this change by automated direct sequencing showed it to be absent.

Table 3.6.

S.No.	Patient	Age at presentation (yrs)	Age of onset	Genetic status	Clinical Status
III:1	Proband	1.3	First year of life	Heterozygous GTG>GTA	Pseudophakic (OD); Posterior sub capsular cataract (OS) and nystagmus
II:5	Father	28	Childhood	Heterozygous GTG>GTA	Aphakic (OU); was operated for both eyes at 5 yrs of age
II:8	Mother	28	Normal	Homozygous GTG>GTG	Myopia (OU)

Table 3.6: Mutational and clinical status of individuals from family with the *GJA3* mutation in family 39

3.2. Single nucleotide polymorphisms (SNP) and benign sequence changes observed

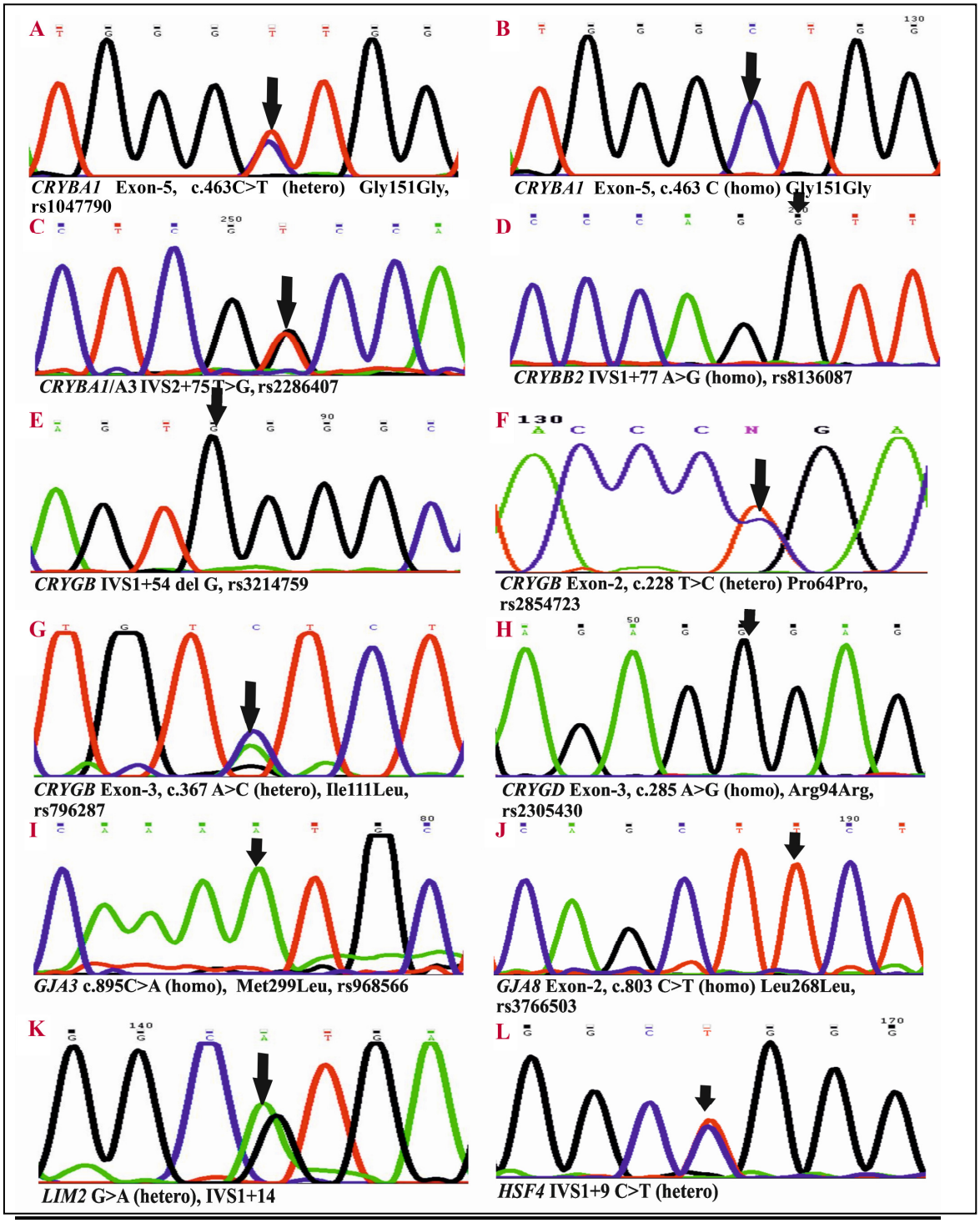
Two heterozygous novel intronic variations were observed in two independent families in *LIM2* (IVS1+14G>A) and *HSF4* genes (IVS1+9C>T). Besides these sequence changes ten previously reported single nucleotide polymorphisms were observed in the cataract genes screened so far (Listed in table 3.7 and Figure(s) 3.7 A-L)

Table 3.7.

S.No.	Family No.	Gene name	Exon	Type of change	Amino acid change	SNP
1	1	*CRYBA1/A3*	5	c.463C>T (homo)	Gly151Gly	rs1047790
	2 & 6	*CRYBA1/A3*		c.463 C>T (hetero)		
2	9	*CRYBA1/A3*	-	IVS2+75 T>G (hetero)	-	rs2286407
3	10	*CRYBB2*	1	IVS1+77 A>G (homo)	-	rs8136087
4	11	*CRYGB*	1	IVS1+54delG	-	rs3214759
5	8	*CRYGB*	2	c.228 T>C (hetero)	Pro64Pro	rs2854723
6	3	*CRYGB*	3	c.367 A>C (hetero)	Met111Ile	rs796287
7	9	*CRYGD*	3	c.285A>G(homo)	Arg94Arg	rs2305430
8	22	*GJA3*	1	c.895 C>A(homo)	Met299Leu	rs968566
9	5	*GJA8*	2	C.803 C>T(homo)	Leu268Leu	rs3766503
10	47	*LIM2*	-	IVS1+14G>A (hetero)	Nil	Novel change
11	11,19 & 21	*HSF4*	-	IVS1+9C>T (hetero)	Nil	Novel change

Table 3.7: Polymorphisms detected in cataract families

Figure(s) 3.7 (A-L)



Figure(s) 3.7 (A-L): Sequences of the observed SNPs (marked by an arrow).

3.3. Analysis of novel pathogenic changes

3.3.1. Multiple sequence alignment

Multiple sequence alignment analysis was done using ClustalW2 software (http://www.simgene.com/ClustalW) for the pathogenic variants Pro197Ser in *GJA3* and Val196Met, Pro199Ser & c.670insA p.203Thr>AsnfsX47 of *GJA8* to show that the residues mutated were found to be highly conserved across species (Figures 3.8A & 3.8B).

Figure 3.8A.

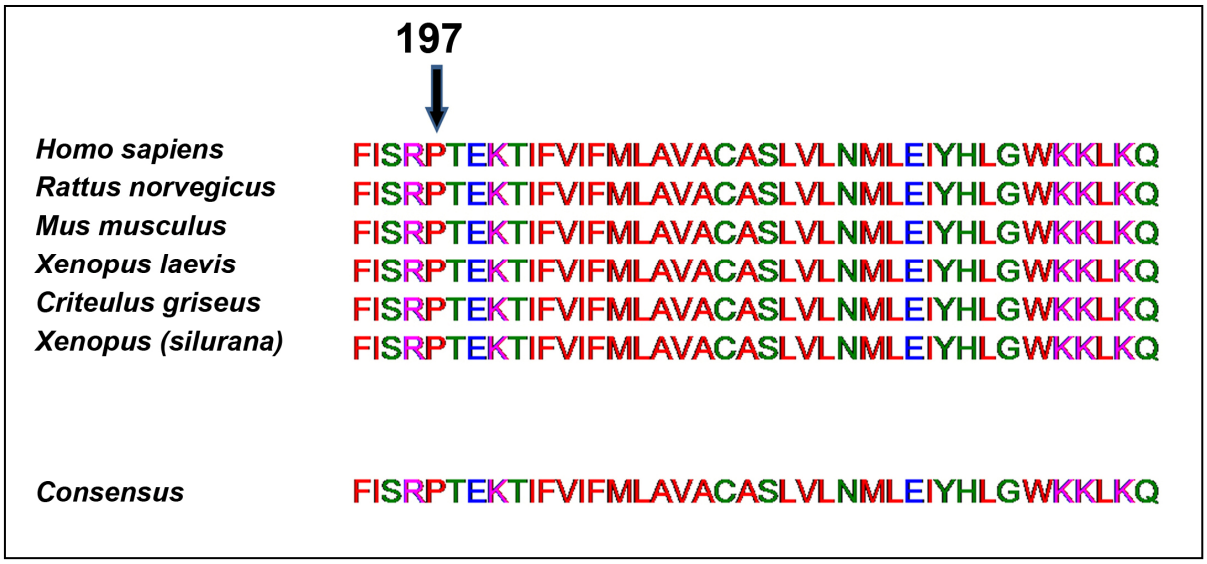


Figure 3.8A: Multiple alignment sequence of GJA3/Cx46 protein the conservation of the Pro199 residue (indicated by an arrow) across species

Figure 3.8B.

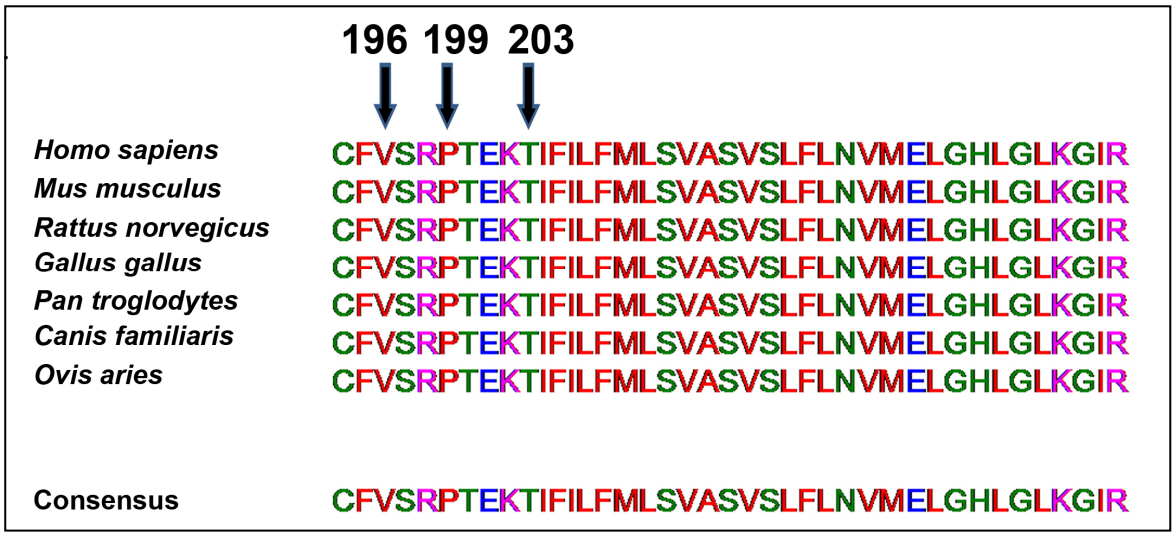


Figure 3.8B: Multiple sequence alignment of GJA8/Cx50 protein with the positions of observed mutations (V196, P199 and T203) marked by arrows

3.3.2. SIFT, Polyphen & HSF analysis

To assess the probable effect of an amino acid substitution on the protein, bio-informatic tools Sorting Intolerant From Tolerant (SIFT), Polymorphism phenotyping (Polyphen) were used.

All the observed pathogenic changes were predicted to have a "probably damaging" effect on protein function except for Val196Met in *GJA8* gene which was found to have a SIFT score of 0.06 thus predicting a probable benign effect on Cx50 protein function (Table 3.8).

Table 3.8.

Gene Name	Mutation identified	Inheritance	PSIC Score difference	Probable effect on the protein	SIFT Score	Probable effect on Protein
GJA8	Pro199Ser	AD	2.696	Probably damaging	0	Yes
GJA8	Val196Met	AR	0.130	Benign	0.06	Tolerated
GJA3	Pro197Ser	AD	2.579	Probably damaging	0	Yes
LIM2	Gly154Glu	AR	2.274	Possibly damaging	0	Yes

Table 3.8: SIFT & PolyPhen scores for the observed missense substitutions

Using the Human splicing finder software, the silent mutation c.84GTG>GTA resulting in Val28Val in the *GJA3* gene and two heterozygous novel intronic variations IVS1+14G>A (*LIM2*) and IVS1+9C>T (*HSF4*) (listed in Table 3.7) were assessed for their possible effect on mRNA splicing.

The c.84GTG>GTA resulting in Val28Val was found to have a significant effect on the mRNA splicing with respect to that of the wild type (WT) sequence. This mutation was predicted to result in the creation of a new potential branch point, with a consensus value (CV) for the WT and the mutant sequence to be at 39.21 and 68.84 respectively, and an enhancer motif site was predicted to be broken with a score of -100.

The novel heterozygous change IVS1+14G>A observed in the *LIM2* gene was predicted to result in the break of a potential donor splice site with a predicted score of 78.88 and 52.04 for the wild type and mutant respectively with a predicted variation of -34.2 suggesting a site broken or disrupted. The enhancer motifs were found predicted to be altered with respect to the WT sequences. The Exonic splicing enhancers (ESE) http://rulai.cshl.edu/tools/ESE2/ (Cartegni, Wang et al. 2003), finder matrices for serine/arginine rich protein (SRp40), SC35, SF2/ASF proteins & RESCUE ESE hexamer (a bioinformatics algorithm developed for identifying sequences with ESE activity, an approach utilized to predict hexamers as candidate ESEs in vertebrate genes (http://genes.mit.edu/burgelab/rescue-ese/)) and EIEs predicted creation of a new enhancer site. Besides this a silencer motif from Sironi *et al* (Sironi, Menozzi et al. 2004) was predicted to be broken with a variation score of 12.1 between wild type and the

mutant Sironi motif. There was no difference between the wild type and the mutant sequence with respect to the creation or destruction of a potential branch point.

The novel heterozygous change IVS1+9C>T of the HSF4 gene was predicted to be only responsible for the creation of new site in the silencer motif , as predicted by IIE from Zhang *et al.*(Zhang and Chasin 2004) but not by any other matrices.

Figure 3.9.

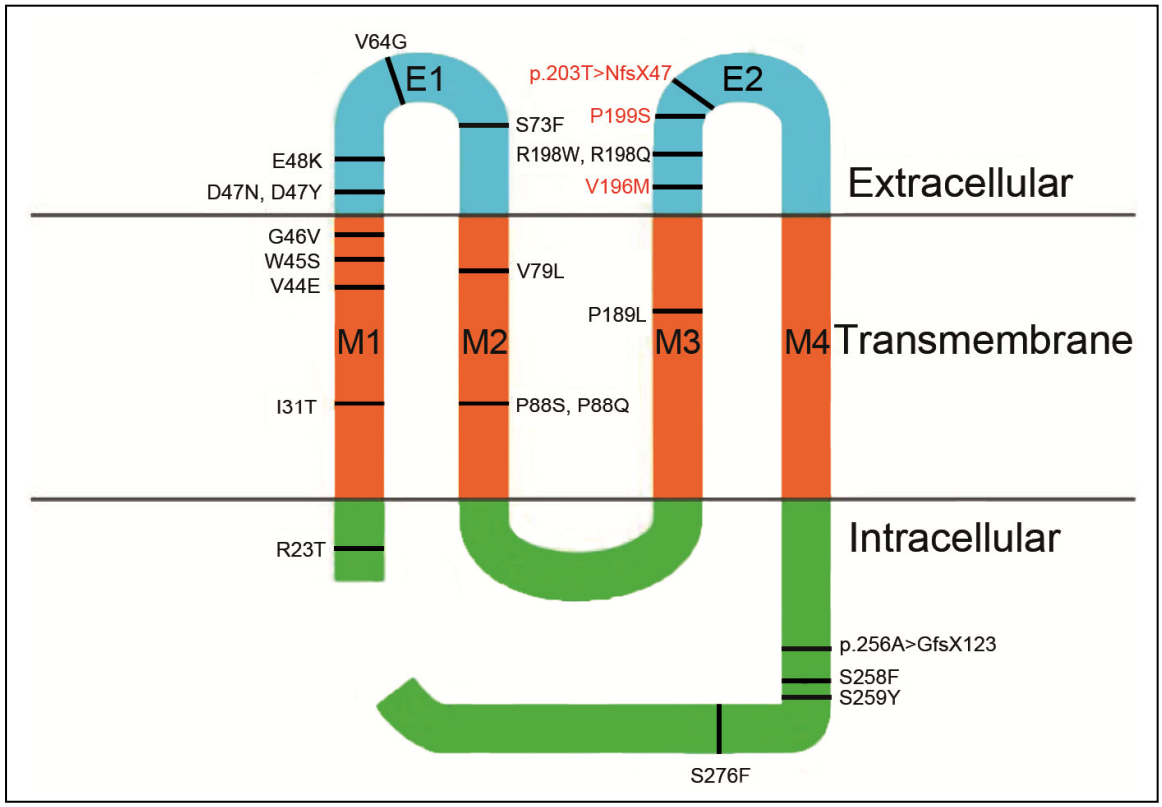


Figure 3.9: Schematic diagram of the Cx50 polypeptide and location of known mutations. Novel mutations observed in our study are highlighted in red colour.

Chapter 4: Results

Prior to our study, properties such as sub-cellular localization, stability and function of pathogenic mutants of GJA8/Cx50 were studied by various research groups using *in vitro* model systems (Berthoud, Minogue et al. 2003; Arora, Minogue et al. 2006; Arora, Minogue et al. 2008). Berthoud *et al.* (Berthoud, Minogue et al. 2003) studied the properties of the wild type and mutant hCx50P88S in stably transfected HeLa, normal rat kidney cells (NRK) or 293 cells and N2A cells. HeLa cells transfected with the hCx50P88S showed cytoplasmic accumulations of Cx50 in addition to its presence in the appositional membranes. The Cx50 protein did not significantly co-localize with other subcellular organelles like endoplasmic reticulum (ER), Golgi apparatus, lysosomes, endosomes or vimentin filaments. Immunoelectronmicroscopic studies localized hCx50P88S to cytoplasmic membrane stacks in close vicinity to the ER and also suggested that the accumulation of hCx50P88S in a cellular structure different from aggresomes. Minogue *et al.* (Minogue, Tong et al. 2009) reported that stable transfection of the *GJA8* gene into the HeLa cells under the control of an inducible promoter (Ponasterone-A) resulted in both hCx50 and hCx50G46V forming gap junctional plaques which were visualized upon fluorescent microscopy.

Arora et al. (Arora, Minogue et al. 2006) reported the sub-cellular distribution of hCx50/GJA8 wild type (wt) and hCx50P88Q mutant in stably transfected HeLa cells by immunoflourescence. They reported that while hCx50 wt was detected at oppositional membranes, the hCx50P88Q was found in the cytoplasm and its appearance varied from diffuse to particulate and, in some cases, to distinct blobs and partially localized at the endoplasmic reticulum and Golgi. Similar defects in localization of the Cx50D47N mutant relative to wild type were found by the same group (Arora, Minogue et al. 2008).

In order to study the alterations in properties of the GJA8/Cx50 mutants that were identified, as described in Chapter 3, as compared to wild type Cx50 (Cx50WT), plasmid constructs of the GJA8 wild-type and mutants (encoding c.670insA p.203Thr>AsnfsX47, Val196Met, Pro199Ser, and Val196Met plus Pro199Ser proteins) were generated as described in Chapter 2. These constructs were transiently transfected into the HeLa cells and their expression and sub-cellular localization of the mutants with respect to that of the Cx50 wt was studied by western blot analysis and immunofluorescence (IF) respectively. The results of the transient expression of the Cx50WT and the mutant cDNA clones in HeLa cells are discussed here.

4.1. Transfections of Cx50WT and mutants followed by detection using IF

Actively dividing HeLa cells were used for transient expression of the *GJA8* cDNA expression plasmids. The transfection procedure was standardized using Cx50WT plasmid. DNA concentrations ranging from 100 ng to 3 µg were transfected and cells were harvested at post-transfection times ranging from 8-24 hrs to compare transfection efficiencies. Since the constructs used had strong CMV promoter, the over-expression of the recombinant protein occurred, leading to accumulation of protein in all cellular compartments along its synthesis-transport pathway. This would prevent assessment of localization of Cx50 mutants and wild type. Addition of cycloheximide (CHX), a potent protein synthesis inhibitor, stops further protein synthesis allowing protein that was already synthesized to be translocated to the membrane. CHX was added to the media at at 21 hours post-transfection and was tested at concentrations ranging from 10-30 µg/ml for 1.5- 6 hrs of exposure, in order to optimize the effect of CHX. Using the optimized

parameters, transfections were initially performed with plasmids Cx50WT and the mutants p.203Thr>AsnfsX47 (frameshift), Val196Met, Pro199Ser, and the double mutant Val196Met+Pro199Ser.

Transient transfection of the constructs in HeLa cells showed detectable levels of the hCx50 wild-type and mutants proteins by immunofluorescence. Expression of Cx50WT and both the missense and the double mutants were observed within vesicle-like structures in the cytoplasm, perinuclear region, as well as at the plasma membrane of HeLa cells. There was no significant difference between the wild-type protein and any of the missense mutants tested (Figures 4.1A, B, C and E). In contrast, the frameshift mutation (203Thr>AsnfsX47) did not localize to the plasma membrane (Figure 4.1 D). Instead it was seen in the cytoplasm in the close vicinity of the ER.

Figure 4.1.

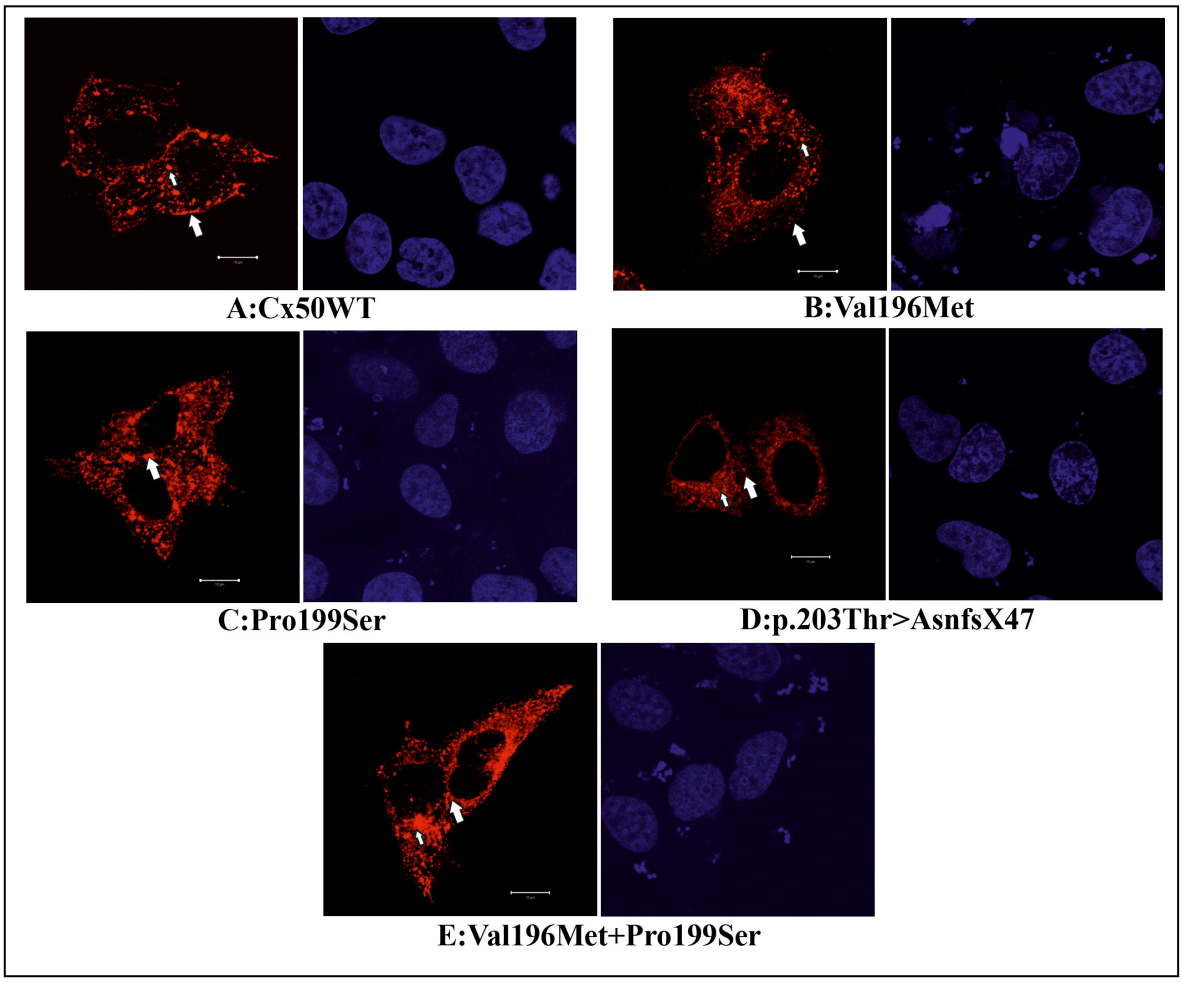


Figure 4.1: Localization of Cx50 wild-type and mutant proteins. Pictomicrographs show the distribution of Cx50WT and mutants (red) in transiently transfected HeLa cells after staining with monoclonal mouse anti-c-myc antibody and Cy-3-conjugated anti-mouse Alexa 488 antibody. Nuclei were counter-stained using DAPI (blue). Bar, 10µm.

4.2. Co-transfection of the hCx50WT and mutants with the VSVG-GFP vector

Localization of Cx50 proteins to the plasma membrane was assessed by co-transfections with the well-characterized temperature-sensitive protein VSVGtsO45 coding plasmid

VSVG-GFP (as described in chapter 2). The VSVG-GFP construct codes for a VSVG-GFP fusion protein, which gets restricted within the endoplasmic reticulum (ER) when the cells are grown at 40ºC due to misfolding. At 32ºC the fusion protein translocates to the plasma membrane. Co-localization of Cx50 proteins with VSVG-GFP was observed after temperature shift to 32ºC in the case of wild-type and missense mutants (Figure 4.2A). Co-transfection of the plasmid encoding the frameshift mutation (203Thr>AsnfsX47), with VSVG-GFP showed that the frameshift mutant was retained within the ER (Fig.4.2A.panel B 3rd row). In addition, ER retention was also seen in the case of VSVG-GFP protein as evident from co-localization of the signals from VSVG-GFP and the Cx50 mutant (Fig.4.2A. panel C 3rd row). Thus it appeared that the frameshift mutant prevents trafficking of VSVG-GFP to the plasma membrane upon temperature shift to 32 ºC.

Figure 4.2.

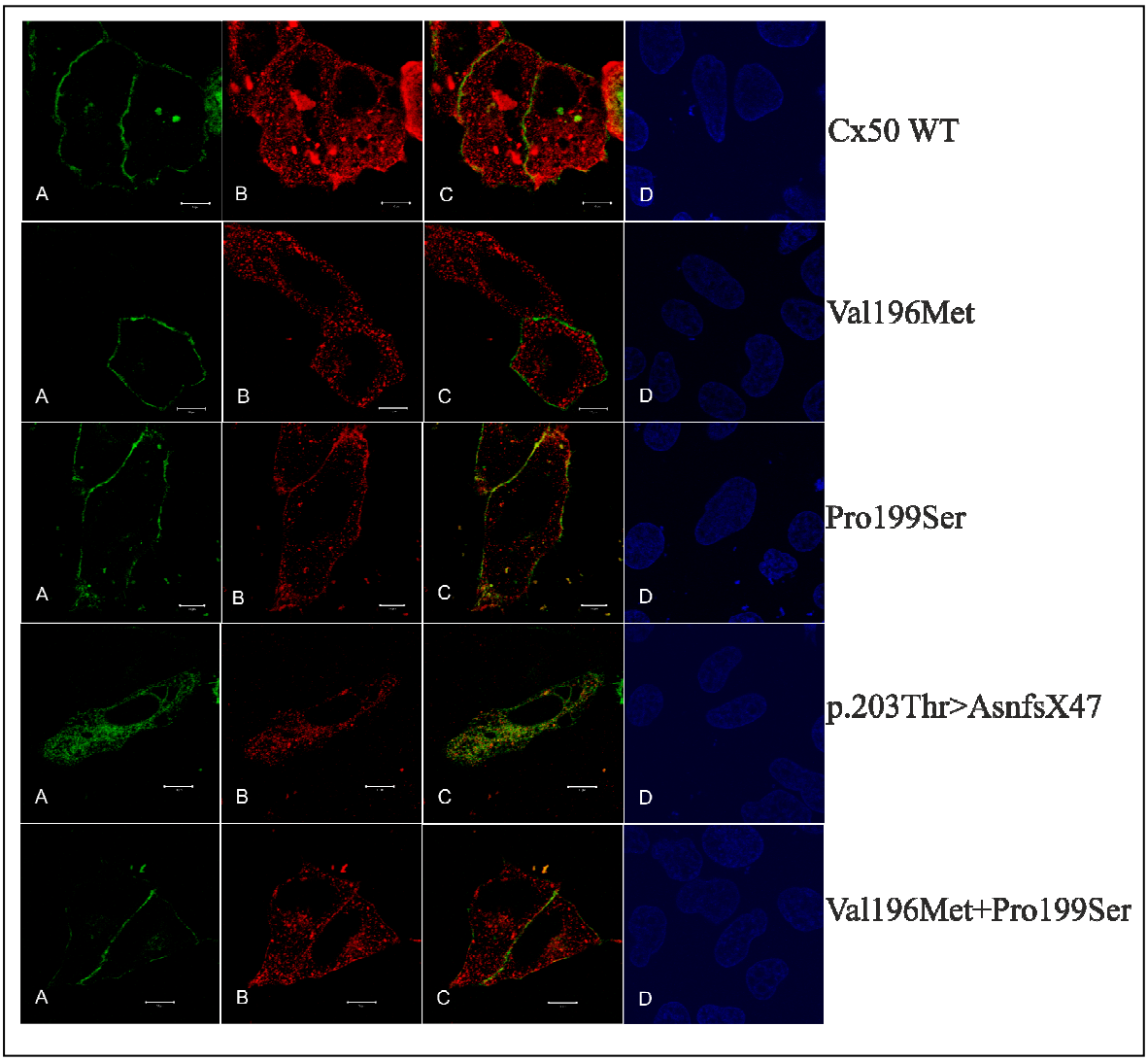


Figure 4.2: Co-localisation of the Cx50 wild-type or mutant proteins along with VSVG-GFP tsO45 construct 24 hrs post-transfection in HeLa cells. Confocal microscope images above show the distribution of VSVG-GFP (A); Cx50 or mutants (red) after staining with monoclonal mouse anti-c-myc primary antibody and Cy-3-conjugated anti-mouse Alexa 488 antibody (B). Overlap of the fluorescent signals in A & B appears yellow in the merged image (C). Nuclei were counter-stained using DAPI (D). Bar, 10 µm.

4.3. Localization of anti-Cx50 immunoreactivity relative to ER marker in transfected HeLa cells

To assess whether the hCx50 mutants Val196Met, Pro199Ser, p.203Thr>AsnfsX47 and Val196Met+Pro199Ser were present in the ER or any other subcellular compartment, we performed immunoflourescence of HeLa cells transiently transfected with the respective cDNA clones using antibodies directed against the ER-specific protein, Calnexin and anti-c-myc epitope of the recombinant hCx50 proteins. No significant co-localization with the ER marker calnexin was observed with either the hCx50WT or any of the missense mutants (upper 3 panels of Figure 4.3). The frameshift mutant was found to co-localize with Calnexin suggesting that the frame-shift mutant was retained within the ER (bottom panel of Figure 4.3).

Figure 4.3.

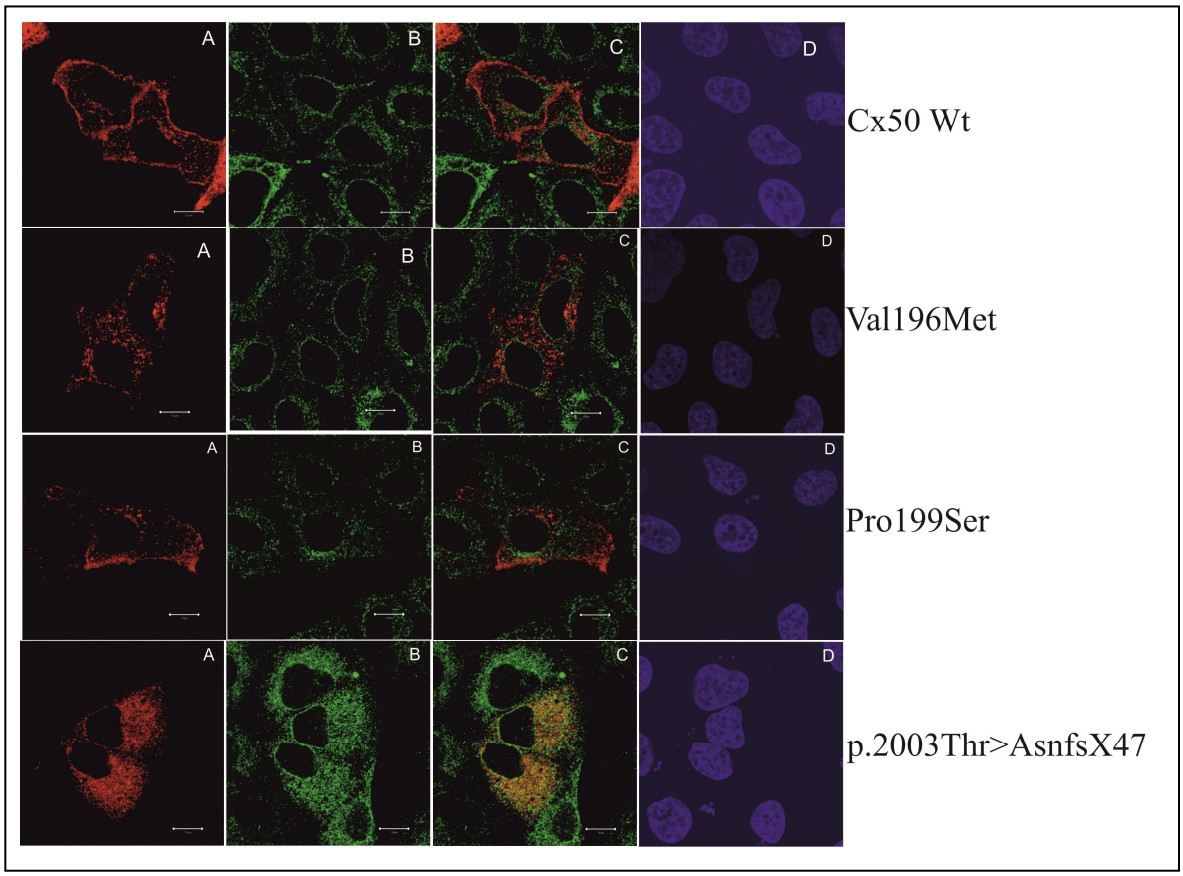


Figure 4.3: Sub-cellular localization of Cx50WT and mutants. Distribution of Cx50WT or mutants after double-immunofluorescence of transiently transfected HeLa cells with monoclonal mouse anti-c-myc antibody (A); and mouse monoclonal anti-Calnexin antibody (B). Merged images are shown in (C). The overlap of the immunofluorescent signals in A and B appears yellow in C. Nuclei were counter-stained using DAPI (D). Bar 10µm.

4.4. Western Blot analysis of Connexin 50 proteins

To assess the size and stability of the wild type hCX50 protein and mutant proteins Val196Met, Pro199Ser, 203Thr>AsnfsX47observed in transfected cells, Western blot analysis was performed as described in chapter 2.

A distinct band of approximately 62 kDa was detected by Western blot using monoclonal mouse anti-c-myc Ab and anti-mouse IgG conjugated with horseradish peroxidise as primary and secondary antibody respectively in HeLa cells transfected with hCX50WT and missense mutants Val196Met, Pro199Ser and double mutant as shown in Figure 4.4., lanes 1, 3, 4 and 5 respectively. As expected, there was no detectable band in lane 6 which was loaded with cell lysate that was transfected with vector alone (negative control). The molecular weight of full length human Cx50 is 62 kDa. The frameshift mutant (p.203Thr>AsnfsX47) was found to be about 35 kDa as shown in lane 2 of Figure 4.4. As an internal control for loading, the blot was probed with antibody against Cdk2 (32 kDa), as shown in the lower panel of Figure 4.4 suggesting that approximately equal amounts of total protein was loaded in all the lanes.

Figure 4.4.

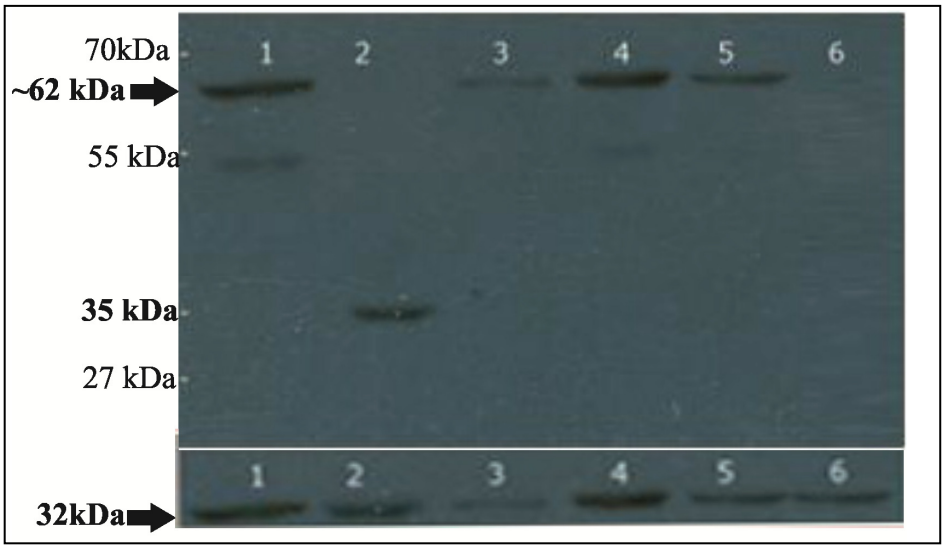


Figure 4.4: Western blot of wild type and mutant Cx50 in transfected HeLa cells. HeLaCx50 WT (lane 1) and missense mutants Pro199Ser (lane 3), Val196Met (lane 4) and the double mutant (lane 5) are shown. The Cx50 frameshift mutant is in lane 2. Positions of molecular mass standards are indicated on the left. The lower panel shows the expression of endogenous cdk2 protein which was used as an internal control.

In a nutshell, on transient transfection of the Cx50/GJA8 cDNA constructs of the Cx50- wt and mutants (Pro199Ser, Val196Met, p.203Thr>AsnfsX47 and the double mutant) in HeLa cells suggested that the Cx50 wt protein was localized to the plasma membrane and was also found within cytoplasmic vesicles as expected. The frameshift mutant was retained within the ER, inhibiting the trafficking of the VSVG-GFP to the plasma membrane. The missense and the double mutants were localized to the plasma membrane, though possibly to a lesser extent when compared to the wild type. Western blot analysis suggested that there was no significant difference in expression levels between the Cx50-wt and the mutants.

Chapter 5: Discussion

From the genetic screening of the 40 families with 30 autosomal dominant (AD) and 10 recessive cataracts (AR) we identified six novel pathogenic changes, three in connexin 50 protein coding *GJA8* gene two in connexion 46 protein coding *GJA3* gene and one in the lens intrinsic membrane protein *LIM2*.

The human lens connexins play an important role in maintenance of the transparency of the lens. Extracellular domains of connexins play an important role both in mediating hemichannel docking (Jiang and Goodenough 1996; Simon and Goodenough 1998) and regulation of voltage gating of the channel (Verselis, Ginter et al. 1994). 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 (White, Bruzzone et al. 1994). Mutations in the *GJA8/Cx50* and *GJA3/Cx46* have been demonstrated to be one of the most frequent reasons for isolated congenital cataracts.

Till date, at least twenty three and fifteen congenital cataract families, including our report (Ponnam, Ramesha et al. 2007) have been linked with *GJA8*and *GJA3* respectively (Graw 2004; Hejtmancik 2008; Wang, Wang et

al. 2009; Zhou, Hu et al 2010 and references therein). Missense and frameshift mutations have been detected in both the genes and significant interfamilial phenotypic variability has been observed. Most mutations reported till date affects the first half of the protein and few mutations have been reported at the carboxy-terminus of the connexin proteins in humans.

Of the three mutations identified in GJA8/Cx50 in this study, one missense mutation Pro199Ser was found to be associated with dominant cataract while the other two, missense mutation Val196Met and frameshift mutation p.203Thr>AsnfsX47 resulting in premature truncation of the protein were associated with recessive cataract. Of all the mutations reported in *GJA8,* five mutations (Val196Met, Arg198Glu, Arg198Trp, Pro199Ser and p.203Thr>AsnfsX47, including all 3 mutations observed in the present study, are predicted to be in the second extracellular domain. Mutations reported in this domain in 2 studies were associated with microcornea in addition to congenital cataracts (Hu, Wang et al. 2010; Devi and Vijayalakshmi 2006).

Similarly, of the fifteen mutations reported in the literature till date in *GJA3,* six missense mutations have been predicted to be in the first transmembrane and two in the second transmembrane domain. The two heterozygous mutations observed in our cohort Val28Val and Pro197Ser are predicted to be in the first and second extracellular domains respectively of the GJA3/Connexin 46 (Cx46) polypeptide. Functional studies on the mutation

of *GJA8 & GJA3* polypeptides have shown diverse mutational mechanisms, such as a dominant negative effect and loss-of-function of the mutant protein (Willoughby, Arab et al. 2003; Arora, Minogue et al. 2006; Arora, Minogue et al. 2008). Functional implications of these mutations may account for the phenotypic differences.

Till date, only two mutations in *LIM2* have been associated with human hereditary cataracts including the one identified in our study. An earlier report by Pras and co-workers (Pras, Levy-Nissenbaum et al. 2002) demonstrated the involvement of *LIM2* mutation Phe105Val leading to hereditary presenile cortical cataract with late onset, pulverulent cortical opacities, and or mild or moderate visual loss. In contrast, all the affected individuals in the family studied by us had congenital cataract evident at or shortly after birth with severe visual impairment as indicated by the presence of nystagmus and amblyopia. It is possible that the mutant Gly154Glu identified in the present study results in loss of function of *LIM2*.

All the missense mutations observed in our study were highly conserved across species and were predicted to affect protein function by SIFT and Polyphen analysis at a varying degree except the Val196Met mutation in Cx50/GJA8, which was predicted to have a 'benign' effect with a score of 0.130 and 0.06 on Polyphen and SIFT analysis respectively. An explanation for the pathogenicity of

this mutation may be that the base substitution alters or creates a splice signal and thereby has more severe effects via alterations in mRNA splicing.

Since all three novel Cx50 mutations observed in our cohort of patients, p.203Thr>AsnfsX47, Val196Met and Pro199Ser were predicted to be in the second transmembrane domain of the Cx50 polypeptide, we attempted to study the functional implications of these mutants by looking at expression and localization of the mutants in transiently transfected HeLa cell lines. Although the Val196Met and Pro199Ser missense mutations were observed in separate families with AR and AD modes of inheritance respectively, since they are in close proximity in the Cx50 polypeptide chain, we generated an artificial double mutant and studied its properties along with the individual mutants. Ectopic expression of the Val196Met, Pro199Ser, artificially generated double mutant Val196Met+Pro199Ser and the frameshift p.203Thr>AsnfsX47 were found to be expressed at similar levels with expected molecular weights on immunoblot assay as compared to the wild type Cx50 protein. Similar to the Cx50WT, the two missense mutants and the double mutant were localized to cell membranes, and in the perinuclear region and within the vesicle like structures in the cytoplasm of the HeLa cells as expected. The two missense mutants localized to the plasma membrane although appeared to do so to a lesser extent than the wild type Cx50. This observation was in contrast to other missense mutations P88S and P88Q wherein the recombinant protein was localized partially at the endoplasmic

reticulum (ER), Golgi and in the cytoplasm as the blobs (Berthoud, Minogue et al. 2003; Arora, Minogue et al. 2006).

The frameshift mutation p.203Thr>AsnfsX47 was found to be restricted to the ER on co-transfection with the VSVGtsO45, and co-localised with calnexin, a marker for ER. In addition, it appeared to prevent the trafficking of VSVG-GFP protein to the plasma membrane. Similar observation was reported in one of the Cx46 frameshift mutation Cx46fs380 wherein the new protein sequence caused by the frameshift contains a retention/retrieval signal that leads to loss of function and retention of the mutant connexin in the cytoplasm (Minogue, Liu et al. 2005). In contrast, DeRosa and co-workers (DeRosa, Mui et al. 2006) described a Cx50 mutant protein with carboxy-terminal truncation at residue 290 that corresponds to physiological truncation occurring during lens maturation. This was found to be expressed and localized to the cell membranes effectively when transiently expressed in HeLa cells.

This study on genetic screening of the candidate genes in an Indian cohort of congenital cataract patients has added to the range of phenotypes and mutations in hereditary cataract. In addition, the present study was the first to report recessive inheritance associated with mutation in *GJA8*/Cx50 and also to identify mutation of the *LIM2* gene in human congenital cataract. The functional studies on the Cx50/*GJA8* wild type and mutants provided an explanation of the impact of the frameshift mutation and indicated a milder degree of impairment in

localization of the missense mutants, providing a possible basis for their pathogenic effect.

Thesis Summary

Cataract is defined as any opacity of the lens resulting in partial or total loss of transparency. Hereditary cataracts are clinically and genetically heterogeneous. Hereditary congenital and juvenile cataracts show Mendelian inheritance. They can be inherited in autosomal dominant (AD) autosomal recessive (AR) or rarely, in X-linked mode. Nineteen genes are associated with autosomal dominant (AD) and 9 genes are associated with recessive inheritance. Hereditary cataracts often present as congenital or developmental cataracts that arise at birth or during the first decade of life, respectively as these opacities can cause blurring of the vision during form vision development, they are clinically very important. Cataracts may account for one-tenth of total blindness in southern India and hereditary cataracts account for about one-fifth of childhood cataracts in this region.

An attempt to identify the causative gene mutation in hereditary congenital cataracts would be useful in knowing the role of those genes with respect to Indian patients. This may in turn lead to a better understanding of the pathogenesis of cataract. The specific aims of this study were - 1) Screening of families with hereditary congenital or developmental cataracts by the candidate gene approach employing single strand conformation polymorphism (SSCP) and automated bi-directional sequencing. 2) To characterize the effects of cataract-causing mutations in Cx50 on the pattern of expression, localization and stability in relation to wild type Cx50 expression in suitable cell lines.

The study protocol was approved by the institutional Review Board of L. V. Prasad Eye Institute, Hyderabad and adhered to the guidelines of the Declaration of Helsinki. Eligible patients (probands) with hereditary cataract either with a diagnosis of congenital or developmental cataract who presented to the Jasti V Ramanamma Children's Eye Care, L. V. Prasad Eye Institute, were recruited for the study with informed consent. A total of 40 families, which comprised of 184 individuals (100 effected and 84 unaffected) with autosomal recessive (AR) and with autosomal dominant (AD) forms of congenital hereditary cataracts were recruited in the study. Of the 40 families recruited 30 were presumed to be with AD and 10 families with AR modes of inheritance. 100 control individuals were included in the study. All control individuals were without any history of congenital hereditary cataract. Blood samples (2-8ml) were collected by simple venipuncture and the genomic DNA was isolated.

In order to fulfil the first aim of our study, 10 candidate genes (6 crystallin genes, 2 connexin genes, the lens intrinsic membrane protein gene (*LIM2*) and the heat shock factor 4 gene (*HSF4*) were screened for mutations in probands from all families.

Genetic analysis revealed 6 sequence changes in the *GJA8, GJA3* and *LIM2* genes in 6 families that were presumed pathogenic mutations. Three of these changes were homozygous and were found in patients with autosomal recessive disease, and three changes were heterozygous, found in patients with autosomal dominant cataract. Homozygous changes found were a homozygous insertion A at codon 203 (c.670insA p.203Thr>AsnFsX47), missense change of c.649G>A (Val196Met) in GJA8/Cx50, and a

missense change c.587 G>A (Gly154Glu) in *LIM2/MP19*. Heterozygous changes found were c.658C>T (Pro199Ser) in GJA8/Cx50, c.589C>T (Pro197Ser) and c.84G>A (Val28Val) in GJA3/Cx46. The 6 mutations mentioned co-segregated with the disease phenotype in each of the families and were absent in al least 50 unrelated ethnically matched normal controls. All the mutations observed were subjected to bioinformatic analysis using SIFT and Polyphen to predict their possible effect on the protein function.

In order to fulfil the 2nd aim of this study, the wild type GJA8/Cx50 coding region was cloned into the mammalian expression vector pcDNA3.1/myc-His and mutants were generated. hCx50-WT and mutant plasmids (Val196Met, Pro199Ser, Val196+Pro199Ser and frameshift p.203Thr>AsnfsX47) were co-transfected with the temperature sensitive GFP-labelled vesicular stomatitis virus G-protein (VSVG-GFP tsO45) encoding plasmid into Hela cells. The cells were grown at 37ºC or at 40ºC for 16 hrs and then at 32ºC till 24 hours post-transfection. Proteins were detected by Western blot (WB) and immunoflourescence (IF) followed by confocal microscopy. The Cx50 WT co-localized to the plasma membrane along with the VSVG-GFP but was also found within cytoplasmic vesicles. The 2 missense mutants and the double mutant (Va19Met+Pro199Ser) localized to the plasma membrane although to a lesser extent than the wild type. The frameshift mutation was retained within the endoplasmic reticulum (ER) and in addition, inhibited trafficking of the VSVG-GFP to the plasma membrane.

In conclusion, key findings of this study are- a) novel mutations were found in 6/40 families with congenital cataracts b) this study identified mutation of the *GJA8* gene

in recessive cataracts for the first time c) the association of *LIM2* gene mutation with autosomal recessive congenital hereditary cataract, was demonstrated for the first time d) missense mutations of GJA8/Cx50 that were studied in vitro displayed milder effects on sub-cellular localization than the frameshift mutant p.203Thr>AsnFsX47, which was retained within the ER and inhibited trafficking of the VSVG-GFP to the plasma membrane. It is possible that the defects of the Cx50 missense mutants can be better appreciated by functional assays for gap junction activity.

Limitations of the Study

Mutation screening of candidate genes was confined to the coding regions as well as flanking intronic regions. In families where no mutations were identified it could be possible that mutations may be present in the deep intronic region or in the promoter regions both of which were not screened in the present study.

Single strand conformation polymorphism (SSCP) technique was employed in gene screening. Although SSCP is one of the most economic and widely used technique in gene screening studies, its sensitivity of detecting a sequence change ranges between 70-90% and hence in families where no mutations were identified it could be possible that genuine sequence change may have been not picked by this technique.

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Appendix

Table 1.2: Genes and mutations associated with hereditary cataracts

Gene Name	Gene Symbol	Chromoso-mal Location	Amino Acid Change	Mode of Inheritance/ tested Population	Phenotype	References
Crystallin, Alpha A	*CRYAA*	21q22.3	W9X	AR/Jewish	Congenital cataract	(Pras, Frydman et al. 2000)
			R12C	AD/Danish & Chinese	Micro-cornea, Cataract	(Hansen, Yao et al. 2007; Zhang, Yam et al. 2009)
			R21W	AD/Danish	Micro-cornea, Cataract	(Hansen, Yao et al. 2007)
			R21L	AD/German	Congenital cataract associated with macular hypoplasia	(Graw, Klopp et al. 2006)
			R49C	AD/Caucasian	Congenital cataract	(Mackay, Andley et al. 2003)
			R54C	AR/ Saudi Arabian	Micro-cornea, Congenital total cataract	(Khan, Aldahmesh et al. 2007)
			G98R	AD/Indian	Pre-senile congenital cataract	(Santhiya, Soker et al. 2006)
			R116C	AD / American	Micro-cornea , Congenital fan-shaped cataract	(Litt, Kramer et al. 1998),
				AD / Indian		(Vanita, Singh et al. 2006)
			R116H	AD/Danish & Chilean	Micro-cornea, Cataract	(Hansen, Yao et al. 2007; Richter, Flodman et al. 2008)
Crystallin, Alpha B	*CRYAB*	11q22.3-q23.1	R11H	AD/Chinese	Congenital nuclear cataract	(Chen, Ma et al. 2009)
			P20S	AD/Chinese	Congenital posterior polar cataract	(Liu, Ke et al. 2006)
			R56W	AR/Saudi Arabian	Juvenile cataract	(Safieh, Khan et al. 2009)
			R120G	French	Desmin related myopathy	(Vicart, Caron et al. 1998)
			R140N	AD/Chinese	Congenital lamellar cataract	(Liu, Zhang et al. 2006)
			150fs	AD/British	Posterior polar cataract	(Berry, Francis et al. 2001)
			A171T	AD/Indian	Congenital cataract	(Devi, Yao et al. 2008)

Crystallin, Beta B1	*CRYBB1*	22q11.2-q12.1	57fs	AR/ Bedouin	Congenital Cataract	(Cohen, Bar-Yosef et al. 2007)
			G220X	AD/American	Congenital pulverulent cataract	(Mackay, Boskovska et al. 2002)
			Q223X	AD/Chinese	Congenital nuclear cataract	(Yang, Zhu et al. 2008)
			S228P	AD/Chinese	Congenital cataract	(Wang, Ma et al. 2007)
			X253R	AD/British	Micro-cornea, Congenital cataract	(Willoughby, Shafiq et al. 2005)
Crystallin, Beta B2	*CRYBB2*	22q11.2-q12.2	S31W	AD/Chinese	Coronary cataract	(Lou, Tong et al. 2009)
			D128V	AD/German	Congenital cataract	(Pauli, Soker et al. 2007)
			R145T	AD/Danish	Hereditary cataracts	(Hansen, Mikkelsen et al. 2009)
			Q147R			
			T150M			
			W151C	AD/Indian	Central nuclear congenital cataract	(Santhiya, Manisastry et al. 2004)
			Q155X	AD/American	Cerulean blue congenital cataract	(Litt, Carrero-Valenzuela et al. 1997)
				AD/Chinese	Progressive polymorphic congenital coronary cataracts.	(Li, Zhu et al. 2008)
				AD/Swiss	"Coppock" like cataract	(Gill, Klose et al. 2000)

			Gene conversion between CRYBB2 and CRYBB2P1 (Q155X)	AD/Chinese	Cerulean cataract family	(Wang, Lin et al. 2009)
			Gene conv. causing C475T (Q155X) and C483T	AD/Indian	Congenital cataract	(Vanita, Sarhadi et al. 2001)
			Q155X	AD/Indian	Inherited pediatric cataract	(Devi, Yao et al. 2008)
			T159X	AD/Danish	Congenital cataract	(Hansen, Mikkelsen et al. 2009)
			V187M	AD/Basotho family	Congenital cataract	(Mothobi, Guo et al. 2009)
Crystallin, Beta B3	CRYBB3	22q11.2-q12.2	G165R	AR/Pakistani	Congenital cataract	(Riazuddin, Yasmeen et al. 2005)
Crystallin, Beta A1	*CRYBA1*	17q11.1-q12	IVS3 G>A splicesite+1	AD/Indian	Cataract, Zonular with sutural opacities	(Kannabiran, Rogan et al. 1998)
				AD/Chinese	Posterior polar cataract	(Gu, Ji et al.)
			IVS3 G>C splicesite+1	AD/Brazillian	Congenital cataract	(Bateman, Geyer et al. 2000)
			IVS2 splice site +1	AD/Australian	Familial cataract	(Burdon, Wirth et al. 2004)
			IVS3+1G>A	AD/Indian	Inherited pediatric cataract	(Devi, Yao et al. 2008)
				AD/Chinese	progressive nuclear and cortical cataracts	(Wang, Wang et al. 2009)
			ΔG91	AD/Swiss	suture-sparing autosomal dominant congenital nuclear cataract	(Ferrini, Schorderet et al. 2004)
				AD/Chinese	Congenital nuclear cataract	(Qi, Jia et al. 2004)
				AD/British	Congenital lamellar cataract	(Reddy, Bateman et al. 2004)

				AD/Chinese	Congenital pulverulent cataract	(Lu, Zhao et al. 2007)
Crystallin, Beta A4	*CRYBA4*	22q11.2-q13.1	L69P	AD/Indian	Bilateral microphthalmia,	(Billingsley, Santhiya et al. 2006)
			F94S	AD/Indian	Lamellar cataract	
Crystallin, Gamma C	CRYGC	2q33-q35	T5P	AD/Candian	Coppock-Like cataract,	(Heon, Priston et al. 1999)
			GlyfsX62	AD/American	Variable zonular Pulverulent cataract	(Ren, Li et al. 2000)
			C109X	AD/Chinese	congenital nuclear cataract	(Yao, Jin et al. 2008)
			W157X	AD/Chinese	Micro-cornea, congenital nuclear cataract	(Lou, Tong et al. 2009)
			R168W	AD/Indian	Congenital cataract	(Santhiya, Shyam Manohar et al. 2002)
				AD/Mexican	Congenital cataract	(Gonzalez-Huerta, Messina-Baas et al. 2007)
				AD/Indian	Inherited pediatric cataract	(Devi, Yao et al. 2008)
Crystallin, Gamma D	CRYGD	2q33-q35	R14C	AD/American	Punctate Cataract, Juvenile Progressive	(Stephan, Gillanders et al. 1999)
				AD/ Chinese	Congenital cataract	(Gu, Li et al. 2006)
			R15S	AD/Chinese	Ccongenital cataract coralliform	(Zhang, Gong et al. 2009)
			P23T	AD/Saudi Arabian	Cerulean and coralliform cataract	(Khan, Aldahmesh et al. 2009)
				AD/Australian	Familial cataracts	(Burdon, Wirth et al. 2004)
				AD/Caucasian	Familial cataracts	(Mackay, Andley et al. 2004)
				AD/ Chinese	Fasciculiform cataract	(Shentu, Yao et al. 2004)
				AD/Chinese	Coralliform cataract	(Xu, Zheng et al. 2004)
			P23S	AD/Russian	Polymorphic congenital cataract	(Plotnikova, Kondrashov et al. 2007)
				AD/ Moroccan	Cerulean congenital cataracts	(Nandrot, Slingsby et al. 2003)
			P24T	AD/Chinese	Congenital coralliform cataract	(Zhang, Gong et al. 2009)

			R36S	AD/Chinese	Nuclear congenital cataract	(Gu, Qi et al. 2005)
			Y56X	AD/Brazilian	Congenital cataracts	(Santana, Waiswol et al. 2009)
			R58H	AD/Canadian	Aculeiform cataract	(Heon, Priston et al. 1999)
				AD/Mexican	Congenital aculeiform cataract	(Zenteno, Morales et al. 2005)
			G61C	AD/Chinese	Coralliform congenital cataracts	(Li, Wang et al. 2008)
			E107A	AD/Mexican	Congenital cataract	(Messina-Baas, Gonzalez-Huerta et al. 2006)
			Y134X	AD/Danish	Congenital cataract	(Hansen, Yao et al. 2007)
			R140X	AD/Indian	Pediatric cataract	(Devi, Yao et al. 2008)
			W156X	AD/Indian	Congenital cataract	(Santhiya, Shyam Manohar et al. 2002)
			G165fs	AD/Chinese	Congenital nuclear cataract.	(Zhang, Yam et al. 2007)
Crystallin, Gamma S	CRYGS	3q27.3	G18V	AD/Chinese	Cortical progressive cataract	(Sun, Ma et al. 2005)
			S39C	AD/Indian	Pediatric cataract	(Devi, Yao et al. 2008)
			V42M	AD/Indian	Congenital cataract	(Vanita, Singh et al. 2009)
Major Intrinsic Protein Of Lens	MIP	12q14	R33C	AD/Chinese	Congenital total cataract	(Gu, Zhai et al. 2007)
			IVS3 -1 G>A	AD/Chinese	Congenital snail-like cataract	(Jiang, Jin et al. 2009)
			V107I	AD/Chinese	Congenital cataracts	(Wang, Jiang et al. 2010)
			E134G	AD/British	Uni-lamellar cataract	(Berry, Francis et al. 2000; Francis, Chung et al. 2000)
			T138R		Multi-focal opacities	
			R233K	AD/Chinese	Binocular polymorphic congenital cataract	(Lin, Hejtmancik et al. 2007; Lin, Wang et al. 2008)
			Δnt3233 (235fs)	AD/ American family of European descent	Congenital cataract	(Geyer, Spence et al. 2006)
			c.607–1G>A	AD/ Chinese		(Wang, Jiang et al. 2010)
Beaded Filament	BFSP2	3q21.2-q22.3	Δ 233Glu	AD/American	Congenital Cataract	(Jakobs, Hess et al. 2000; Zhang, Gao et al. 2006)

Structural Protein-2			E233Δ	AD/Chinese	Progressive congenital Cataract	(Cui, Gao et al. 2007)
					Congenital Y-sutural cataract	(Zhang, Guo et al. 2004)
			R287W	AD/American	Juvenile-onset progressive cataract	(Conley, Erturk et al. 2000)
			R339H	AD/China	Congenital lamellar cataracts	(Shiels, Bennett et al. 2008)
Beaded Filament Structural Protein-1	BFSP1	20p11.23	Δ3343bp	AR/Indian	Juvenile- onset cataract	(Ramachandran, Perumalsamy et al. 2007)
Connexin 50	GJA8	1q21.1	R23T	AD/Iranian	Progressive congenital nuclear cataract	(Willoughby, Arab et al. 2003)
			I31T	AD/Chinese	Congenital nuclear cataract	(Wang, Wang et al. 2009)
			V44E	AD/Indian	Microcornea, congenit developmental catar	(Devi and Vijayalakshmi 2006)
			W45S	AD/Indian	Micro-cornea and bilateral congenital cataract	(Vanita, Singh et al. 2008)
			G46V	AD/Palestinian	Congenital total cataract	(Minogue, Tong et al. 2009)
			D47N	AD/British	Inherited cataract	(Arora, Minogue et al. 2008)
			D47Y	AD/Chinese	Congenital cataract	(Lin, Liu et al. 2008)
			E48K	AD/Pakistani	Zonular nuclear pulverulent cataract	(Berry, Mackay et al. 1999)
			V64G	AD/Chinese	Nuclear cataract	(Zheng, Ma et al. 2005)
			S73F	AD/Danish	Congenital cataract	(Hansen, Mikkelsen et al. 2009)
			V79L	AD/Indian	‘‘Full Moon’’ With Y-Sutural Opacity	(Vanita, Hennies et al. 2006)
			P88S	AD/British	Zonular pulverulent cataract	(Shiels, Mackay et al. 1998)
			P88Q	AD/British	Lamellar pulverulent cataract	(Arora, Minogue et al. 2006)
				AD/Indian	Balloon–like cataract with Y- sutural opacities	(Vanita, Singh et al. 2008)
			P189L	AD/Danish	Micro-cornea, Congenital cataract	(Hansen, Yao et al. 2007)
			V196M	AR/Indian	Congenital cataract	Ponnam & co-workers, unpublished

			R198Q	AD/Indian	Congenital or Developmental Cataract with micro-cornea	(Devi and Vijayalakshmi 2006)
			R198W	AD/Chinese		(Hu, Wang et al. 2010)
			P199S	AD/Indian	Total cataract	Ponnam & co-workers, unpublished
			T203NfsX47	AR/Indian	Total cataract	(Ponnam, Ramesha et al. 2007)
			A256GfsX123	AR/German	Triangular cataract	(Schmidt, Klopp et al. 2008)
			S258F	AD/Chinese	Congenital nuclear cataract	(Gao, Cheng et al. 2010)
			S259Y	AD/Danish	Congenital cataract	(Hansen, Mikkelsen et al. 2009)
			S276F	AD/Chinese	Pulverulent nuclear cataract	(Yan, Xiong et al. 2008)
Connexin 46	GJA3	13q11-13q12	D3Y	AD/Hispanic	Zonular pulverulent cataract	(Addison, Berry et al. 2006)
			L11S	AD/Danish	Congenital "Ant-Egg" Cataract	(Hansen, Yao et al. 2006)
			V28M	AD/Indian	Hereditary congenital cataract	(Devi, Reena et al. 2005)
			F32L	AD/Chinese	Nuclear pulverulent cataract	(Jiang, Jin et al. 2003)
			R33L	AD/Indian	Congenital cataract	(Guleria, Sperling et al. 2007)
			V44M	AD/Chinese	Congenital nuclear catarct	(Zhou, Hu et al. 2010)
			W45S	AD/Chinese	Progressive nuclear cataract	(Ma, Zheng et al. 2005)
			P59L	AD/Caucasian	Nuclear punctuate cataract	(Bennett, Mackay et al. 2004)
			N63S	AD/British	Zonular pulverulent cataract	(Mackay, Ionides et al. 1999)
			R76H	AD/Australia	Congenital cataract with incomplete penetrance	(Burdon, Wirth et al. 2004)
			R76G	AD/Indian	Hereditary congenital cataract	(Devi, Reena et al. 2005)
			T87M	AD/Indian	Congenital pearl box like cataract	(Guleria, Vanita et al. 2007)
			P187L	AD/British	Congenital zonular pulverulent cataracts	(Rees, Watts et al. 2000)
			N188T	AD/Chinese	Congenital nuclear pulverulent cataract	(Li, Wang et al. 2004)
			S380QfsX476	AD/British	Zonular pulverulent cataract	(Mackay, Ionides et al. 1999)

			Pro197Ser	AD/Indian	Developmental lamellar cataract	Ponnam & co-workers, unpublished
Transmembrane protein	TMEM114	16p13.3	t(16;22)(p13.3; q11.2) balanced translocation	AD/British (Caucasian)	Congenital lamellar cataract	(Jamieson, Farrar et al. 2007)
Eph-receptor type-A2	*EPHA2*	1p36.13	G948W	AD/American with English origin	Posterior polar cataracts	(Shiels, Bennett et al. 2008)
			A785T	AR/Pakistani	Congenital nuclear cataract	(Kaul, Riazuddin et al. 2010)
			T940I	AD/Chinese	Posterior polar cataract	(Zhang, Yam et al. 2009)
			c.2915_2916delTG	AD/British	Posterior polar cataract	
			c.2826-9G>A	AD/Australian	Total congenital cataract	
V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog; Maf	MAF	16q23.1	R288P	AD/British(Caucasian)	Cataract, Ocular anterior dysgenesis and coloboma	(Jamieson, Munier et al. 2003)
			K297R	AD/Indian	Cerulean cataract	(Vanita, Singh et al. 2006)
			L320E	AD/Danish		(Hansen, Mikkelsen et al. 2009)
Paired-Like Homeodomain Transcription Factor 3	PITX3	10q25	S13N	AD/American	Congenital cataract and anterior segment mesenchymal dysgenesis (ASMD)	(Semina, Ferrell et al. 1998)
			650ΔG	AD/Hispanic	Posterior polar cataract	(Berry, Yang et al. 2004)
			650ΔG	AD/Lebanese	Posterior polar cataract and neuro-developmental abnormalities	(Bidinost, Matsumoto et al. 2006)
			656ins17	AD/American	Congenital cataract and ASMD	(Semina, Ferrell et al. 1998)
Heat-Shock Transcription Factor 4	HSF4	16q21-q22.1	A20D	AD/Chinese	Infantile cataract	(Bu, Jin et al. 2002; Hansen, Mikkelsen et al. 2009)
			I87V	AD		

			L115P	AD/Chinese	Lamellar cataract	
			R120C	AD/Danish	Marner cataract	
			c.1327+4A-->G	AR/Tunisian	Congenital total white cataract	(Smaoui, Beltaief et al. 2004)
			R74H	AD/Chinese	Congenital total white cataract	(Ke, Wang et al. 2006)
			A175P & c.595_599delGGGCC	AR/ Pakistani	Congenital Cataracts	(Forshew, Johnson et al. 2005)
Galactokinase	GALK1	17q25.1	p.G137fsX27	AR/Pakistan	Nuclear hereditary congenital cataract	(Yasmeen, Riazuddin et al. 2010)
			L139P			
Chromatin modifying protein-4B	*CHMP4B*	20q11.21	D129V	AD/Caucasian	Progresssive childhood posterior subcapsular cataract	(Shiels, Bennett et al. 2007)
			E161K			
Glucosaminyl (N-Acetyl) Transferase 2, I-Branching Enzyme	GCNT2	6p24-p23	W328X or W326X	AR/Arab families	Congenital cataracts	(Pras, Raz et al. 2004)
			G348Q,R383H	Taiwanese		(Yu, Twu et al. 2001)
Lens Intrinsic Membrane Protein 2 (19kDa)	LIM2	19q13.4	F105V	AR/ Iraqi Jewish family	Presenile cataract	(Pras et al. 2002)
			G154E	AR/Indian	Congenital cataracts with nystagmus	(Ponnam, Ramesha et al. 2008)

Table 2.1: List of the primer sequences used with the annealing conditions and restriction enzymes used if any

Gene name	Primer's name	Sequence (5'→3')	Length of Oligo (bp)	Product Size (bp)	Annealing Conditions	Cycles	Restriction enzyme used
CRYAA	**CRYAA-1F**	ATCCCTTAATGCCTCCATTC	20	407	61C/30sec/ 2mM Mg^{+2}	35	***Nla III***
	CRYAA-1R	GAGCAAGACCAGAGTCCATC	20				
	CRYAA-2F	ACCGAAGCATCTCTGTTCTG	20	257	61C/30sec/ 1.5mM Mg^{+2}	35	
	CRYAA-2R	CCTCTCCCACCTCTCAGTG	19				
	CRYAA-3F	GGCAGCTTCTCTGGCATGGGG	21	321	63^{o}C/25sec /1mM Mg^{+2}	33	***Sau3AI***
	CRYAA-3R	AGGGCTGCAGGGGAGCCAGCC	21				
CRYBA1/A3	**CBA-1F**	GGCAGAGGGAGAGCAGAGTG	20	207	58^{o}C/30sec /1mM Mg^{+2}	35	
	CBA-1R	CACTAGGCAGGAGAACTGGG	20				
	CBA-2F	AGTGAGCAGCAGAGCCAGAA	20	293	58^{o}C/30sec /1mM Mg^{+2}	35	
	CBA-2R	GGTCAGTCACTGCCTTATGG	20				
	CBA-3F	AAGCACAGAGTCAGACTGAAGT	22	269	57^{o}C/30sec /2mM Mg^{+2}	35	
	CBA-3R	CCCCTGTCTGAAGGGACCTG	20				
	CBA-4F	GTACAGCTCTACTGGGATTG	20	357	59^{o}C/30sec /1mM Mg^{+2}	35	
	CBA-4R	ACTGATGATAAATAGCATGAACG	23				
	CBA-5F	GAATGATAGCCATAGCACTAG	21	290	59^{o}C/30sec /1.5mM Mg^{+2}	35	
	CBA-5R	TACCGATACGTATGAAATCTGA	22				
	CBA-6F	CATCTCATACCATTGTGTTGAG	22	295	57^{o}C/30sec /2.0mM Mg^{+2}	35	
	CBA-6R	GCAAGGTCTCATGCTTGAGG	20				
CRYBB2	**CBB-2F1**	TCTGTGGGCATTTGCTGACCC	21	300	59^{o}C/30sec /1mM Mg^{+2}	38	
	CBB-2R1	GCTAACAGCATTGAAGTCTCTGCCC	25				
	CBB-2F2	GGGACAGTCTGAAACCAGGA	20	285	59^{o}C/30sec /1.5mM Mg^{+2}	38	
	CBB-2R2	ATGCCAAGCCCATTTTACAG	20				

	CBB-2F3	ACCCTTCAGCATCCTTTGG	19	314	59°C/30sec /1.5mM Mg^{+2}	38	
	CBB-2R3	GCAGACAGGAGCAAGGGTAG	20				
	CBB-2F4	CCAGGATTCTGCCATAGGAA	20	273	59°C/30sec /1.0 mM Mg^{+2}	38	
	CBB-2R4	CTCCAAGGTGGCAGAGAGAG	20				
	CBB-2F5	GAGTGATGTGTGGGACATGC	20	308	59°C/30sec /1.0 mM Mg^{+2}	38	
	CBB-2R5	CCCCAGAGTCTCAGTTCCTG	20				
	CBB-2F6	GCCTCTCTCTCTGTCTGCTTCT	22	398	59°C/30sec /1.0 mM Mg^{+2}	35	
	CBB-2R6	TCACACACACACACACACAC	22				
CRYGD	**CRYGD-1F**	CAGCAGCCCTCCTGCTAT	18	229	59°C/30sec /1.0mM Mg^{+2}	40	
	CRYGD-1R	TCGTAGAGGGTGATCTGCAA	20				
	CRYGD-2F	GGGGGCCAGAGACTGGGGCAGGA	23	344	60°C/30sec /1mM Mg^{+2}	38	
	CRYGD-2R	CCTGAGGGCCTGGGTCCTGAC	21				
	CRYGD-3F	CACACTTGCTTTTCTTCTCTTTT	23	341	55°C/30sec /1.5 mM Mg^{+2}	35	
	CRYGD-3R	TTAAGAAACAACAAAAGAGGAC	22				
CRYGB	**CRYGB-1AF**	TACAGTGACGTTCCCGCAGT	20	298	62°C/15sec /1.25 mM Mg^{+2}	30	
	CRYGB-1AR	ATAGATCATCCAGCAGCCGC	20				
	CRYGB-2BF	GGGCCGCAGCTACGAATG	18	330	57°C/30sec /1.25 mM Mg^{+2}	30	***Sau3AI***
	CRYGB-2BR	CCACCCTGGGGGACAGAGC	19				
	CRYGB-3AF	GCCAGGGAGGTGTAGGGACT	20	280	60°C/30sec /1.25 mM Mg^{+2}	30	
	CRYGB-3AR	CCCCCGGCCACAGCAGATAC	20				
	CRYGB-3BF	GGGCAGCTGGATCCTCTATG	20	268	64°C/30sec /1.25 mM Mg^{+2}	30	
	CRYGB-3BR	GCTGAGTGCCATTATCACTTA	21				
CRYGC	**CRYGC-1AF**	CCCCATCACACTGAACTCG	19	265	64°C/30sec /1.25 mM Mg^{+2}(2.5 pm)	30	
	CRYGC-1AR	GCAGCCGCTCTCCACCCG	18				
	CRYGC-2AF	GCCGCAGCTACGAAACCAC	19	289	57°C/30sec /1.25 mM Mg^{+2}	32	
	CRYGC-2AR	GCAAACCTCCCTCCCTGTAA	20				
	CRYGC-3AF	TTGGTTGGACAAATTCTGGAAG	22	279	57.5°C/30sec/1.25 mM Mg^{+2}	32	
	CRYGC-3AR	CTGGCACCGCCTGTACTCT	19				

	CRYGC-3BF	GGGCTGCTGGGTCCTCTAC	19	294	57°C/30sec /1.25 mM Mg^{+2}	32	
	CRYGC-3BR	CCCCACCCCATTCACTTCT	19				
GJA3	**GJA3-1AF**	TGTTCATGAGCATTTTCCTC	20	146	56°C/30sec /1.25mM Mg^{+2}	35	
	GJA3-1AR	CTGTCGTAGCAGACGTTCTC	20				
	GJA3-1BF	GAGCAGTCAGACTTCACCTG	20	213	58°C/30sec /1mM Mg^{+2}	32	
	GJA3-1BR	CTCTCTCTTCAGCTGCTCCT	20				
	GJA3-1CF	GCATGGAAGAGAAGAAGAAAG	21	220	57°C/30sec /2mM Mg^{+2}	32	
	GJA3-1CR	TCGAAGCCGTACAGAAAGTA	20				
	GJA3-1DF	TCATCTTCAAGACGCTGTTC	20	239	57°C/30sec /2mM Mg^{+2}	35	
	GJA3-1DR	CTGCTTGAGCTTCTTCCAG	19				
	GJA3-1EF	GTCCCTGCTGCTCAACAT	18	269	51°C/30sec /1mM Mg^{+2}	35	
	GJA3-1ER	TCAGGGCTAGCAGTTTGAA	19				
	GJA3-1FF	GGTTCCCACCCTACTATGC	19	186	51°C/30sec /1mM Mg^{+2}	35	
	GJA3-1FR	CCCAGTTCTGCTCAGTCAT	19				
	GJA3-1GF	AGTCCGCCAAGCTCTACA	18	226	51°C/30sec /1mM Mg^{+2}	35	
	GJA3-1GR	CTCCCCTCCAGACTGCT	17				
	GJA3-1HF	CCACTCGCGCACGAGG	16	268	49°C/30sec /1mM Mg^{+2} 10%DMSO	35	
	GJA3-1HR	AAGATCACTACACAGCT	17				
GJA8	**GJA8-1F**	AGGGCCAATCCAGTCATA	18	277	55°C/30sec /1.5mM Mg^{+2}	35	
	GJA8-1R	TGGCAACCTTTTTTCCTTT	18				
	GJA8-2AF	CGCGTTAGCAAAAACAGA	18	288	55°C/30sec /2.5mM Mg^{+2}	35	
	GJA8-2AR	TCGTAGCAGACGTTCTCG	18				
	GJA8-2BF	GGATGAGCAATCCGACTT	18	293	54°C/30sec /1.0mM Mg^{+2}	35	
	GJA8-2BR	CCAGCCGGAACTTCTTAG	18				
	GJA8-2CF	ACCAGGGCAGCGTCAA	16	270	54°C/30sec /1.0mM Mg^{+2}	35	
	GJA8-2CR	CAGAGGCCACAGACAA	16				
	GJA8-2DF	CCACGGAGAAAACCATCT	18	253	54°C/30sec /1.0mM Mg^{+2}	35	
	GJA8-2DR	TCGGTCAAGGGGAAATAG	18				
	GJA8-2EF	CTGTCTCCTCCATCCAGAA	19	204	54°C/30sec /1.5mM Mg^{+2}	35	
	GJA8-2ER	CGTAGGAAGGCAGTGTCTC	19				

	GJA8-2FF	TCAGTTCGAGGAGAAGATCA	20	254	50°C/30sec /1.0mM Mg^{+2}	35	
	GJA8-2FR	TTTCACCCTCCTTATCCACT	20				
	GJA8-2GF	GGAGCAGGAGAAGGTG	16	287	49°C/30sec /1.0mM Mg^{+2} 10% DMSO	35	
	GJA-2GR	TTCCTTTCATCTTGCC	16				
LIM2	**LIM-1F**	CCATTGTGTAGGGAGGCTTA	20	216	52°C/30sec /1.0mM Mg^{+2}	35	
	LIM-1R	AGGTCCTGGGAGAAGAAGG	19				
	LIM-2AF	CAGTTCCTCCCTTCAAGTCC	20	159	53°C/30sec /1.0mM Mg^{+}	35	
	LIM-2AR	ACTGCATCCAGTGGTCTGTT	20				
	LIM-2BF	TGTACAGCTTCATGGGTGGT	20	255	52°C/30sec /1.0mM Mg^{+2}	35	
	LIM-2BR	TGGAATACAGGTGTCCTTGG	20				
	LIM-2CF	TACCTGCAGACAGACAGCAT	20	238	52°C/30sec /1.0mM Mg^{+2}	35	
	LIM-2CR	CCCAACTTAACCTTCAAACC	20				
	LIM-3F	TCATCTCAGAGGTAGCAGCA	20	279	52°C/30sec /1.0mM Mg^{+2}	35	
	LIM-3R	ATTGGGGTTTGAGATGAGAG	20				
	LIM-4F	AAAATCACACCCAGCCTTAG	20	248	52°C/30sec /1.0mM Mg^{+2}	35	
	LIM-4R	ACTCTATCTGCTGCCCACTC	20				
	LIM-5F	GGTGTTGGGCTCTCTTG	17	231	51°C/30sec /1.0mM Mg^{+2}	35	
	LIM-5R	CTAGGAACCAGGATTTCA	18				
HSF4	**HSF-1F**	TTCCGCGGCTTTGACGAG	18	257	51.5°C/30sec/1.5mM Mg^{+2} +10% DMSO	35	
	HSF-1R	GGGCATGGGTGTTCACT	17				
	HSF-2F	AGAGTGAGCATGAGTGTGT	19	273	58°C/30sec /1.0mM Mg^{+2}	35	
	HSF-2R	ATCCCCGGCACCGAGTC	17				
	HSF-3F	AACATGTGTGAGTCCCTACG	20	260	52°C/30sec /1.0mM Mg^{+2}	35	
	HSF-3R	CTCCTCCTCTTTGCTCATTC	20				
	HSF-4F	GAATGAGCAAAGAGGAGGAG	20	267	50°C/30sec /1.0mM Mg^{+2}	35	
	HSF-4R	GTTATGGTCTCATCCCGAAC	20				
	HSF-5F	AACTCTCAGATGCCTCAGC	19	297	52°C/45sec /1.0mM Mg^{+2}	38	
	HSF-5R	CAAAGAGACACTGGATCAGC	20				
	HSF-6F	GGCGTGTGCCAAAGTATGAA	20	199	52°C/30sec /1.0mM Mg^{+2}	35	
	HSF-6R	GAAGGAGTTTCCAGAGGAGA	20				
	HSF-7F	GACTGACACCCTAGCAACAG	20	299	51°C/30sec /1.5mM Mg^{+2}		
	HSF-7R	AGGAACAGGCTCTCCATAAG	20				
	HSF-8F	TGTTGGTGGGGTTCTGGCT	19	257	54°C/30sec /1.5mM Mg^{+2}	30	
	HSF-8R	TACAGCCATCTGGGCTGCCA	20				
	HSF-9AF	TGGCTGTAGGGGTAGAGG	18	161	50°C/30sec /1.0mM Mg^{+2}	35	
	HSF-9AR	CGCAGAAGTCACACTCGT	18				
	HSF-9BF	CTGGCACTGCTCAAAGAA	18	281	54°C/30sec /1.5mM Mg^{+2}	35	
	HSF-9BR	GCTTTGTGGGCTGGTAAG	18				

	HSF-10F	GATGCATCTGGGTTCCTT	18	270	59°C/30sec /1.0mM Mg^{+2}	35	
	HSF-10R	GAGCTCTGGGTCAGTGGT	18				
	HSF-11F	TGGTGGGGTTCTAGCTCTC	19	121	52°C/30sec /1.5mM Mg^{+2}	35	
	HSF-11R	ACAGAGAACAGCTATGGA	18				
	HSF-12F	GTGATTTCCCAGCTGTCC	18	204	51°C/30sec /1.0mM Mg^{+2}	35	
	HSF-12R	ACTCAATCAAGGTAGGTCA	19				
	HSF-13F	GAAGAAAGGAGGGGGAAC	18	267	59°C/30sec /1.0mM Mg^{+2}	35	
	HSF-13R	AAGCCAAGAAGGATGTGC	18				

List of Publications, Awards & Presentations

- Ponnam SP, Ramesha K, Tejwani S, Matalia J, Kannabiran C. A missense mutation in LIM2 causes autosomal recessive congenital cataract. Mol Vis. 2008, 14:1204-8. PMID: 18596884

- Ponnam SP, Ramesha K, Tejwani S, Ramamurthy B, Kannabiran C. Mutation of the gap junction protein alpha 8 (GJA8) gene causes autosomal recessive cataract. J Med Genet. 2007; 44(7):e85. PMID: 17601931

- Awarded the **best scientific poster** titled "Molecular Genetic analysis of congenital hereditary cataracts" in July 2008 at the Indian Eye Research Group held at Madurai, TN, India.

- Qualified the National level **ICMR**-Junior research fellowship (JRF) entrance examination and was awarded the fellowship in the year 2004.

- Qualified the National level **CSIR** Junior Research Fellowship Examination & SRF fellowship in the year 2005 and 2006 respectively

- Qualified the **GATE** 2004 examination

- Selected for the oral scientific oral presenter award at the national conference titled **"GENOMICS: IMPACT ON HUMAN HEALTH"** held in 2007 organized by Dept. of Zoology, O.U and AP Akademi of sciences, Hyderabad.

- Presented scientific posters at various international conferences including Asia-ARVO 2009 held at Hyderabad, India, and Fifth international symposium on Genetics, Health and Disease titled **"OMICS in the 21st Century"** 2008 held at G.N.D.University, Amritsar, Punjab, India.

- Presented scientific oral presentations at the various national conferences including 75th Society of Biological Chemists (India) held at JNU, New Delhi and at Indian Eye Research Group (**IERG**) at Hyderabad.

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A missense mutation in *LIM2* causes autosomal recessive congenital cataract

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Purpose: To identify mutations in the *LIM2* gene in families with hereditary congenital or juvenile-onset cataract.
Methods: Forty families (total of 100 affected and 84 unaffected individuals) were recruited for the study. Probands were screened for pathogenic alterations in 10 different candidate genes including the lens intrinsic membrane protein-2 (*LIM2*) gene. Exons and flanking regions were screened by polymerase chain reaction (PCR) amplification, single-strand conformation polymorphism, and sequencing. Sequence changes were evaluated in 75 unrelated normal controls.
Results: A missense mutation, Gly154Glu, was found in *LIM2* in one family with four individuals diagnosed with autosomal recessive cataract from two generations. An evaluation of seven individuals (four affected and three unaffected) showed that the mutation was homozygous in the affected members and heterozygous in unaffected members tested. It was absent in 75 unrelated ethnically matched normal controls. All affected individuals had a severe phenotype of congenital cataracts and visual impairment.
Conclusions: The Gly154Glu mutation involves a non-conservative change that presumably results in loss of function of the MP19 protein. This study shows the involvement of *LIM2* in human congenital cataract.

Blindness due to cataract represents a major cause of treatable blindness in different parts of the world [1-3]. Cataract in infants and children carries the risk of irreversible visual loss or amblyopia due to improper visual inputs to the brain, and cataract-related amblyopia accounts for 8% of blindness in children in southern India [4]. Loss of transparency of the lens can be a result of multiple factors that cause changes in the cellular or macromolecular organization in the lens, which result in light scattering and opacification. Hereditary cataracts arising from single gene mutations have provided insights into some of the structural and functional requirements for lens transparency. So far, 16 genes are identified to cause autosomal dominant congenital cataract [5], and eight genes are identified for autosomal recessive cataract [5-8].

The lens intrinsic membrane protein-2 gene (*LIM2*) encodes an abundant integral lens membrane protein of 19 kDa, MP19 (alternatively known as MP17/MP18/MP20). The function of MP19 is not clearly understood as yet. It localizes to junctional regions of the lens fiber cell membrane as well as throughout fiber cell membranes, suggesting a role in lens junctional communication [9,10]. MP19 has been shown to be absent from proliferating epithelial cells in the lens with expression becoming prominent in differentiating cells as well as in mature lens fiber cells [11,12]. It binds calmodulin [13, 14] as well as galectin, a protein associated with lens cell membranes [15]. While various models have been proposed for MP19 topology, it is predicted to have four transmembrane segments and two extracellular [16,17] with NH_2- and COOH-termini in the cytoplasm or with the NH_2-terminus integrated into the membrane [18].

Evidence for the role of *LIM2* in cataract came initially from the *To3* mouse, an ENU (ethyl nitroso-urea)-induced mutant that demonstrated a semi-dominant cataract that mapped to the same region as *Lim2* on mouse chromosome 7 [19]. While both heterozygous and homozygous mutants had dense cataracts, homozygotes also had microphthalmia, disorganization of lens fibers, and rupture of the lens capsule [19,20]. The *To3* mutation was identified as a Gly15Val change in *Lim2* [20]. A similar cataract phenotype was also reproduced in transgenic mice carrying the *Lim2* transgene with the same mutation [21]. *Lim2* homozygous knockout mice were found to have pulverulent nuclear opacities and altered refractive properties of the lens whereas heterozygotes had normal lenses [22], suggesting that loss of function of *Lim2* is responsible for the phenotype. To date, there has been one report of human hereditary cataract arising from a mutation in *LIM2* in a family with autosomal recessive presenile cortical cataract with a missense mutation, Phe105Val [23].

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Table 1. Details of polymerase chain reaction primers for *LIM2* amplification.

Exon	Primer name	Primer sequence (5′-3′)	Product size (bp)	Annealing temp (°C)
I	LIM 1F	CCATTGTGTAGGGAGGCTTA	213	52
	LIM 1R	AGGTCCTGGGAGAAGAAGG		
II	LIM 2AF	CAGTTCCTCCCTTCAAGTCC	159	53
	LIM 2AR	ACTGCATCCAGTGGTCTGTT		
	LIM 2BF	TGTACAGCTTCATGGGTGGT	255	52
	LIM 2BR	TGGAATACAGGTGTCCTTGG		
	LIM 2CF	TACCTGCAGACAGACAGCAT	238	52
	LIM 2CR	CCCAACTTAACCTTCAAACC		
III	LIM 3F	TCATCTCAGAGGTAGCAGCA	279	52
	LIM 3R	ATTGGGGTTTGAGATGAGAG		
IV	LIM 4F	AAAATCACACCCAGCCTTAG	248	51
	LIM 4R	ACTCTATCTGCTGCCCACTC		
V	LIM 5F	GGTGTTGGGCTCTCTTG	231	51
	LIM 5R	CTAGGAACCAGGATTTCA		

Sequences of forward (F) and reverse (R) primers used for amplification of *LIM2* exons are shown above along with sizes of PCR products for each primer pair. Exon 2 was amplified in three fragments using primer sets denoted 2A-2C.

Here, we report a homozygous missense mutation in *LIM2* in a family with autosomal recessive cataract of congenital onset.

METHODS

Patients and sample collection: Probands presenting at the pediatric ophthalmology clinic of our institution with a diagnosis of congenital or developmental cataract were recruited for the study based on an ophthalmic evaluation by slit lamp biomicroscopy of probands and available family members by two independent ophthalmologists. Inclusion criteria were the presence of a bilateral cataract of congenital or developmental type based on history and/or examination. Exclusion criteria were a history of trauma, unilateral (non-familial) cataract, co-existing ocular disease, mental retardation, microcephaly, cerebral palsy, systemic syndromes, and maternal history of intrauterine infections or antenatal steroid use. After prior approval of the protocol by the Institutional Review Board of the L.V. Prasad Eye Institute (Hyderabad, India), informed consent was obtained from participants, blood samples (2-8 ml) from the probands and family members were collected in heparin-coated vacutainers by venipuncture, and family history, pedigree, and clinical data were recorded. Forty families were recruited as part of the study. They consisted of 30 families with dominant cataracts and 10 families with recessive cataracts with a total of 100 affected and 84 unaffected individuals.

Mutational analysis: Genomic DNA was extracted from blood leukocytes by the standard phenol-chloroform method. Ten candidate genes (six crystallin genes, two connexin genes, *LIM2*, and the *heat shock factor 4* (*HSF4*) gene) were screened for mutations in probands from all families. The gene sequences were retrieved from the Ensembl database. Suitable primers for polymerase chain reaction (PCR) amplification of exons and flanking sequences of *LIM2* were designed using primer design software. Primer sequences used are as shown in Table1. PCR was performed by using 50 ng of DNA template, 5 pmol each of forward and reverse primers, 1 unit of Taq DNA polymerase (Bangalore Genei, Bangalore, India), and 0.2 mM dNTPs in a total volume of 25 µl. Cycling conditions were as follows: initial denaturation at 94 °C for 2 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 51–53 °C for 30 s, and elongation at 72 °C for 45 s followed by one cycle of final extension at 72 °C for 8 min.

PCR products were mixed with two volumes of formamide. Samples were denatured by heating at 95 °C for 5 min and then snap chilled. The products were then subjected to electrophoresis on 8% polyacrylamide gels containing 5% glycerol. All samples were electrophoresed at room temperature and at 4 °C. The variants observed on single-strand conformation polymorphism (SSCP) analysis were identified by bi-directional dideoxy sequencing of the relevant PCR products using fluorescent automated methods. Screening for the observed mutation or variation was performed on DNA from 75 ethnically matched, unrelated normal controls using SSCP. Family members of the proband were tested to check for cosegregation of the sequence change with disease. Designation of sequence changes is according to the cDNA sequence of human *LIM2*. Human *LIM2* sequences were obtained from the Vega genome browser; human transcript ID OTTHUMT00000151603 and gene ID OTTHUMG00000071186. The mouse *Lim2* genomic sequence (gene ID ENSMUSG000000046134) was from Ensembl.

To assess the probable effect of an amino acid substitution on the protein, SIFT (Sorting Intolerant from

Tolerant) scores were obtained by using SIFT software. SIFT aligns sequences homologous to the protein of interest from the databases and predicts whether a specific amino acid substitution will be tolerated by calculating normalized probabilities (range from 0 to 1) for each substitution at a particular position. Scores below a threshold of 0.05 are predicted to be deleterious and those above the threshold are predicted as tolerated [24,25].

RESULTS

Screening of probands from 40 families for pathogenic mutations revealed one mutation in *LIM2* in one family (pedigree in Figure 1). There were four affected individuals from two generations (IV:2, IV:3, V:5, and V:6 in Figure 1). All four individuals were offspring of consanguineous marriages. In addition to the four affected members, three other unaffected relatives-the mother (IV:5), the father (III:8), and the maternal grandfather (II:4) of the proband-were evaluated.

Sequence analysis showed a change of c.587G>A in human *LIM2* cDNA (Figure 2) involving a codon change of GGG>GAG, which leads to a predicted glycine-154 to glutamic acid substitution (Gly154Glu). All four affected individuals in the family were homozygous for the mutant allele (Figure 2, bottom), and the three unaffected individuals tested were heterozygous (Figure 2, top). The change was absent in 75 ethnically matched normal controls.

The clinical features of the members analyzed are presented in Table 2. The proband presented to us at six years of age and was pseudophakic in both eyes with dense amblyopia. He had reportedly been affected since the age of three years, at which time he had cataract surgery. His visual acuity was 6/60 (20/200) in the right eye and counting fingers at 1 m in the left eye. His affected sibling was reported to have cataract since he was two years old. He was four years old at presentation and when he had cataract surgery. The proband's maternal aunt and uncle also had congenital and developmental cataract respectively and were operated for cataract removal within the first decade of their lives (Table 2). All affected individuals had nystagmus and dense amblyopia.

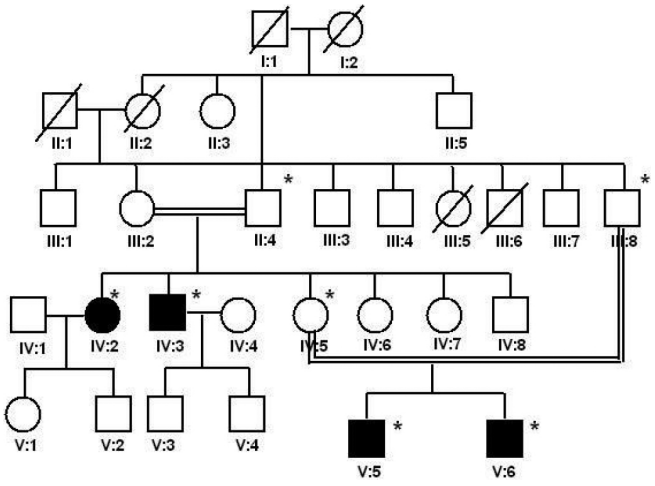

Figure 1. Pedigree of family with autosomal recessive cataract. Dark circles and squares represent affected females and males, respectively, and open symbols represent unaffected individuals. Symbols with an asterisk show individuals on whom genetic analysis was done.

DISCUSSION

The mutation described here is the second known human mutation in *LIM2* causing hereditary cataract. It differs from the Phe105Val mutation reported by Pras and coworkers [23] in that the associated phenotype reported in their study was relatively mild with a late-onset of cataract, pulverulent cortical opacities, and mild or moderate visual loss. All affected individuals in the family studied by us had congenital cataracts evident at or shortly after birth with severe visual impairment as indicated by the presence of nystagmus and amblyopia. The absence of a phenotype in heterozygous carriers points to a loss of function of the Gly154Glu mutant MP19 protein as a possible cause of the disease. A substantial impact of the Gly154Glu mutation on protein structure is suggested by the non-conservative nature of the substitution, involving the replacement of a neutral, polar small amino acid (glycine) by a charged, larger amino acid (glutamic acid). Further, introduction of a charged amino acid into this position, which is predicted to be located within the fourth

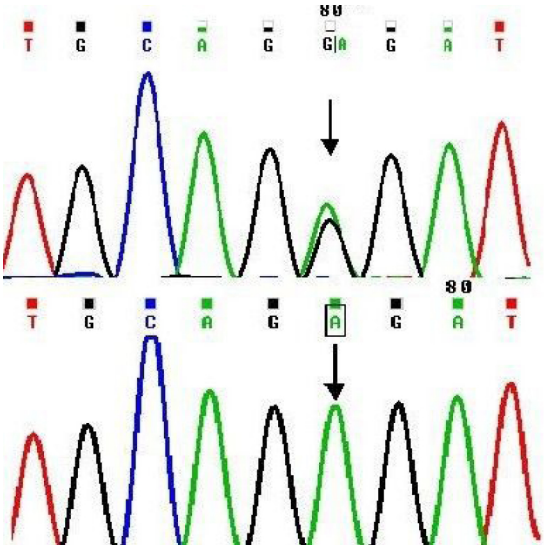

Figure 2. Sequence electropherogram showing mutation in *LIM2*. Heterozygous c.587G>A change (arrow) found in unaffected members of the family (see Figure 1) involving GGG>GAG (Gly154Glu) codon change is shown in the top panel. The bottom panel shows homozygous G>A at this position in affected individuals (arrow at site of mutation).

Table 2. Mutational and clinical status of individuals from family with the *LIM2* mutation.

Individual	Relation with proband	Age at presentation	Age at onset	Genotype	Clinical status
V:5	Proband	6 years	3 years	GGG>GAG (homozygous)	Total cataract, surgery at 3 years, amblyopia
V:6	Sibling	4 years	1.5 years	GGG>GAG (homozygous)	Total cataract, surgery at 4 years, poor fixation, amblyopia
IV:2	Maternal aun	35 years	Birth	GGG>GAG (homozygous)	Surgery at 3 years, nystagmus, amblyopia
IV:3	Maternal uncle	29 years	<8 years	GGG>GAG (homozygous)	Surgery at 10 years, nystagmus, amblyopia
III:8	Father	35 years	-	GGG>GAG (heterozygous)	Normal
IV:5	Mother	27 years	-	GGG>GAG (heterozygous)	Normal
II:4	Maternal grandfather	55 years	-	GGG>GAG (heterozygous)	Normal

Pedigree of the family is shown in Figure 1. The numbers for each individual in the left column are according to the pedigree drawing.

transmembrane segment of MP19 [16], would be presumably incompatible with the structure and topology of the protein in its wild type form. It is possible that the mutant may not be targeted to the membrane thus resulting in loss of function.

We used the SIFT tool [25] to assess the probable impact of the Gly154Glu substitution as compared with the Phe105Val found in human cataract [23] and the Gly15Val in *To3* mouse [20]. This action yielded a score of 0 for the Gly154Glu mutation, thereby interpreting it as deleterious to protein function. A deleterious effect is also predicted for the mouse Gly15Val mutation, which gave a score of 0.03. On the other hand, the Phe105Val substitution has a SIFT score of 0.24, predicting that it would be tolerated. The predicted low impact of the Phe105Val mutation on the protein is compatible with the mild cataract observed in the individuals carrying the mutation [23]. However, the dominant effect of the *To3* (Gly15Val) mutant is not explainable from existing data. A dominant negative effect of the *To3* mutation was also suggested in studies on the *Lim2* ^{To3} transgenic mice, which demonstrated severe cataracts despite the amount of transgenic *Lim2* mRNA present being much lower than wild type *Lim2* mRNA [21]. Chen and coworkers [26] have shown that the MP19 *To3* protein accumulates within the cytoplasm of transfected cells while the wild type MP19 localizes to the plasma membrane. They proposed that MP19 *To3* is a deleterious gain of function mutant that might be cytotoxic in lens fibers. In contrast, homozygous *Lim2* knockout mice had relatively mild changes consisting of pulverulent central opacities and changes in refractive properties of the lens [22]. Comparison of these latter observations with the recessive mutation identified in our study may imply a different role for MP19 in mice and humans. The effect of these mutations on the MP19 protein cannot be gauged fully until its function and physiological effects are understood.

The location of the Gly154 codon spans two exons including bases at the 3′ boundary of exon 4 and 5′ boundary of exon 5. The second base of the codon, which is the site of the G>A mutation, is located at the 5′ end of exon 5 next to the splice acceptor site. This raises the possibility that this mutation might have an additional effect on mRNA splicing, which needs to be investigated further.

The present study provides further evidence for the importance of *LIM2* in maintaining the normal structure and function of the lens and demonstrates for the first time that a mutation in *LIM2* results in human autosomal recessive congenital cataract.

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Mutation of the gap junction protein alpha 8 (GJA8) gene causes autosomal recessive cataract

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ONLINE MUTATION REPORT

Mutation of the gap junction protein alpha 8 (*GJA8*) gene causes autosomal recessive cataract

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J Med Genet 2007;**44**:e85 (http://www.jmedgenet.com/cgi/content/full/44/7/e85). doi: 10.1136/jmg.2007.050138

Background: *GJA8* encodes connexin-50, a gap junction protein in the eye lens. Mutations in *GJA8* have been reported in families with autosomal dominant cataract.
Objective: To identify the disease gene in a family with congenital cataract of autosomal recessive inheritance.
Methods: Eight candidate genes were screened for pathogenic alterations in affected and unaffected family members and in normal unrelated controls.
Results: A single base insertion leading to frameshift at codon 203 of connexin 50 was found to co-segregate with disease in the family.
Conclusions: These results confirm involvement of *GJA8* in autosomal recessive cataract.

Cataract is defined as any opacity of the lens resulting in partial or total loss of transparency. Hereditary cataracts are clinically and genetically heterogeneous, often presenting as congenital or developmental cataracts that arise at birth or during the first decade of life, respectively. As these opacities can cause blurring of the vision during form vision development, they are clinically very important. Cataracts may account for about one-tenth of total childhood blindness in Southern India[1] and hereditary cataracts account for about one-fifth of childhood cataracts in this region.[2] The majority of hereditary cataracts that have been genetically characterised to date are of autosomal dominant inheritance.[3] Mutations in six genes (*CRYAA*,[4] *LIM2*,[5] *GCNT2*,[6] *HSF4*,[7] *CRYBB3*,[8] and *BFSP1*[9]) have been associated with the autosomal recessive cataracts.

MATERIALS AND METHODS

The study protocol was approved by the institutional review board of the L. V. Prasad Eye Institute and followed the tenets of the Declaration of Helsinki. A family of southern Indian origin was recruited for the study. The proband and five available family members underwent a complete ophthalmic evaluation and blood samples were obtained after informed consent. Diagnosis of hereditary cataract was based on the presence of a bilateral familial lenticular opacity of any size of congenital or developmental type (based on history/examination and age of onset <16 years) as evaluated independently by two examiners. Patients with a history of trauma, or having unilateral (nonfamilial) cataract, co-existing ocular disease, mental retardation, microcephaly, cerebral palsy, systemic syndromes, or a maternal history of intrauterine infections or antenatal steroid use were excluded.

Genomic DNA was isolated from peripheral blood leukocytes using standard protocols. The gene sequence for *GJA8* was retrieved from the Ensembl database (ENSG00000121634). Primers were designed using the Primer 3 software (http://frodo.wi.mit.edu) for PCR amplification of the coding region of *GJA8*, which is present in exon 2. Seven pairs of overlapping primers were used to obtain fragments of <300 bp in length for single-strand conformation polymorphism (SSCP) analysis (listed in table 1). PCR products were mixed with two volumes of formamide, denatured by heating at 90℃, snap-chilled and loaded onto 8% non-denaturing polyacrylamide gels with 5% glycerol. All samples were subjected to electrophoresis at 4℃ and at room temperature. Gels were fixed and subsequently stained with silver nitrate, and DNA visualised under visible light. Variants on the SSCP were subjected to bidirectional sequencing by automated methods. Screening for the observed mutations was performed on 75 ethnically matched unrelated normal controls by SSCP.

RESULTS AND DISCUSSION

The proband (IV: 1 in fig 1) presented at our institution at 12 years of age. She had a history of poor vision, white opacities in both eyes and nystagmus since birth. She had undergone cataract surgery elsewhere, and had an unaided visual acuity in both eyes of counting fingers at about 1 metre. Her brother (IV: 2 in fig 1), who was similarly affected, had a history of decreased vision since birth and on examination, had total cataracts, nystagmus and a visual acuity in both eyes of counting fingers. The pedigree obtained upon examination of all available members of the family was suggestive of autosomal recessive inheritance (fig 1).

We employed a candidate gene approach consisting of SSCP-based screening and sequencing. We screened eight genes including six crystallin genes and two connexin genes to identify mutations. Screening of the coding regions of *GJA8* revealed a single base insertion causing a frameshift at codon 203 (c.670insA; p.Thr203AsnfsX47; shown in fig 2) that was

Table 1 Primers used for amplification of *GJA8*

Name of primer	Primer sequence (5′ to 3′)
GJA8-2A(F)	CGCGTTAGCAAAAACAGA
GJA8-2A(R)	TCGTAGCAGACGTTCTCG
GJA8-2B(F)	GGATGAGCAATCCGACTT
GJA8-2B(R)	CCAGCCGGAACTTCTTAG
GJA8-2C(F)	ACCAGGGCAGCGTCAA
GJA8-2C(R)	CAGAGGCCACAGACAA
GJA8-2D(F)	CCACGGAGAAAACCATCT
GJA8-2D(R)	TCGGTCAAGGGGAAATAG
GJA8-2E(F)	CTGTCTCCTCCATCCAGAA
GJA8-2E(R)	CGTAGGAAGGCAGTGTCTC
GJA8-2F(F)	TCAGGTCGAGGAGAAGATCA
GJA8-2F(R)	TTTCACCCTCCTTATCCACT
GJA8-2G(F)	GGAGCAGGAGAAGGTG
GJA-2G(R)	TTCCTTTCATCTTGCC

Primers used for amplification of the coding regions of GJA8 within the second exon are listed above. F and R in parentheses refer to forward and reverse primers respectively.

Abbreviation: SSCP, single-strand conformation polymorphism

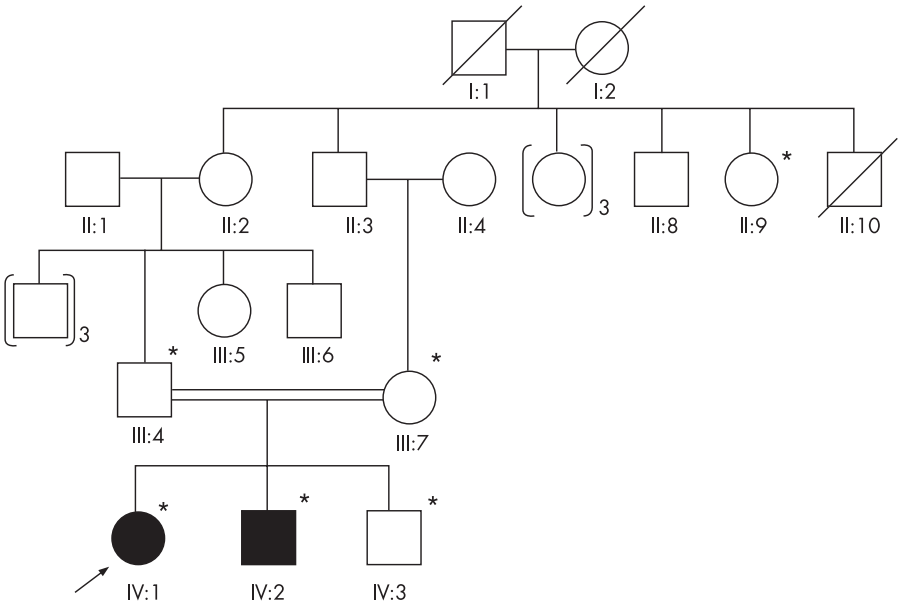

Figure 1 Pedigree of family with autosomal recessive cataract. The dark symbols indicate affected individuals and open symbols indicate unaffected individuals. Symbols marked with asterisks represent individuals who were analysed.

homozygous in the two affected members IV:1 and IV:2 (fig 1), and heterozygous in the parents (III:4 and III:7 in fig 1), sibling (IV:3 in fig 1) and a second-degree relative of the proband (II:9 in fig 1), all of whom were unaffected. This change was not found in 75 unrelated controls. The mutation is predicted to result in a frameshift at codon 203 with a stop codon after 46 amino acids of altered reading frame, producing a truncated protein consisting of 248 amino acid residues (fig 3).

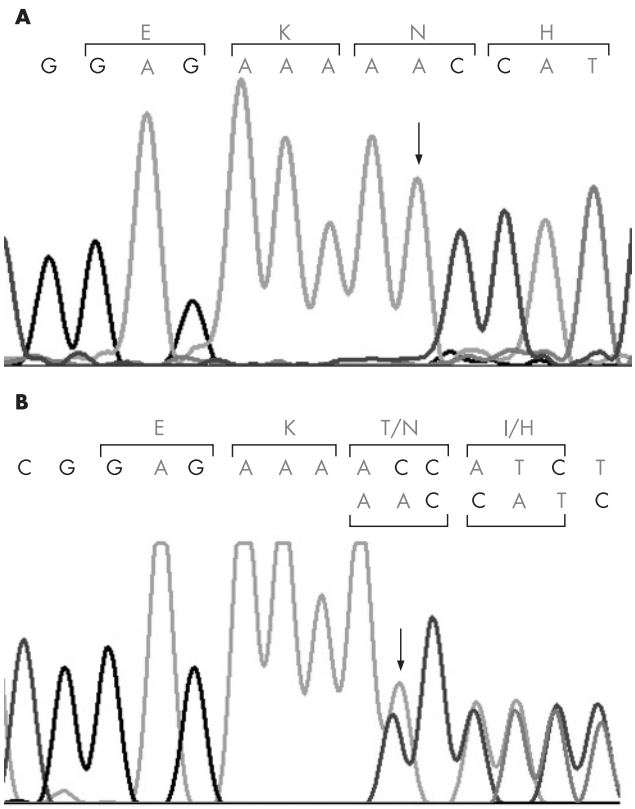

Figure 2 Sequence of the *GJA8* coding region. The sequence of *GJA8* showing the insertion (arrow) of an A at position 670 of the cDNA (c.670insA) (A) homozygous in the proband and (B) heterozygous in unaffected parent. Codons are marked by brackets and amino acids indicated above. Codons and amino acids for both wild type and mutant alleles are shown in (B).

GJA8 encodes the gap junction protein connexin 50 (Cx50), which is one of the major lens connexins along with connexins 43 (locus *GJA1*) and 46 (locus *GJA3*). Connexins 50 and 46 are expressed in differentiating lens fibres and persist in mature fibres, and connexin 43 is expressed in lens epithelial cells.[10–14] Connexins form intercellular channels consisting of two halves or hemichannels, the connexons, each made up of six connexin monomers. Mutations in *GJA3* and *GJA8* are known to result in autosomal dominant cataract. Eight different mutations have been reported in the *GJA8* gene (table 2), all of which are missense changes.

The insertion described here is located in codon 203, which is predicted to be in the second extracellular domain of connexin 50; a frameshift at this position would be expected to lead to the disruption of the C-terminal half of the protein (amino acids 203–433) and thereby produce a functionally null allele. Possible consequences could be instability or non-functionality of the mutant protein, or degradation of the mRNA through the nonsense-mediated decay pathway. A mechanism of disease involving loss of function at connexin loci has also been suggested in mouse models of recessive cataract. *GJA3* or *GJA8* homozygous knockout mice are reported to have a cataractous phenotype, whereas heterozygous knockout mice ($GJA3^{+/-}$, $GJA8^{+/-}$) have normal lenses.[15 16]

DeRosa *et al*.[17] studied the properties of Cx50 proteins with C-terminal truncations at residue 290 that correspond to physiological truncations occurring during lens maturation. Such truncated Cx50 proteins were found to be expressed and localised to the cell membrane effectively when transiently expressed in HeLa cells.[17] Interestingly, they also retained the ability to form channels, but had significantly impaired conductance compared with wild-type connexin 50.[17] Truncation at residue 290 would be expected to result in loss of the C-terminal cytoplasmic domain of the protein (residues 228–433),[18] with all putative transmembrane domains intact.

In comparison, the mutation identified in the present study would be predicted to result in the loss of the second extracellular domain and the subsequent transmembrane and cytoplasmic domains. Heterologous expression in cell lines and

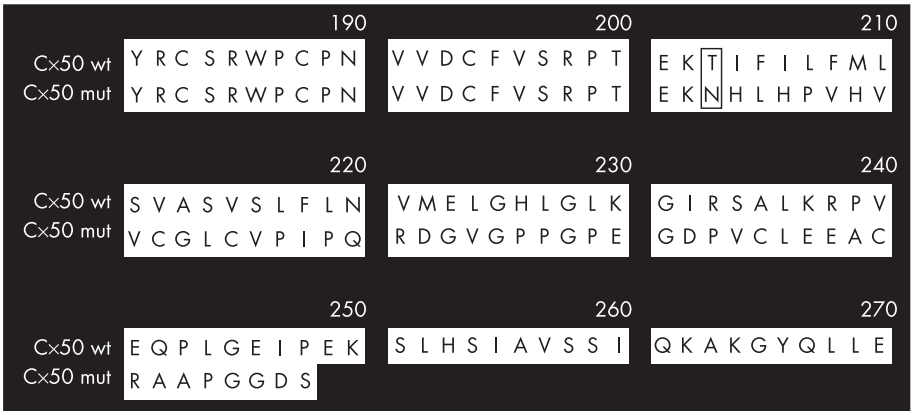

Figure 3 Sequences of wild type and mutant *GJA8* proteins. Partial protein sequences of the wild type *GJA8*/Cx50 (wt) and predicted sequence of the insertion mutant (mut) (c.670insA, p.Thr203AsnfsX47) are shown. The residue (position 203) at the start of the frameshift is boxed. The mutant protein terminates at 248 amino acids. Residues are numbered with respect to the wild-type Cx50 sequence.

in *Xenopus* oocytes would be required to determine the level of inactivation of the protein.

Studies on various mutant connexin proteins causing dominant cataract in humans and mice, have suggested varied mechanisms of action. Dominant negative effects have been proposed for the *GJA8* mutant proteins Pro88Ser[19 20] and Pro88Gln[21] based on studies in *Xenopus* oocytes. Studies on the effect of the *GJA8* mutation Gly22Arg (found in *Lop10* mice), in mouse lenses also revealed dominant negative effects.[22] In that study, the mutant proteins were found to interfere with the formation of gap-junction channels. In contrast, other mutants of both *GJA3* and *GJA8*, when tested in *Xenopus* oocytes, have been observed to result in loss of function without any dominant negative effects. These are the *GJA8* mutant Asp47Ala (D47A) in the *No2* mice[23] and two *GJA3* mutants Asn63Ser and fs380, causing cataract in humans.[24] Yet another mechanism of action suggested for the *GJA3* (Cx46) fs380 mutant,[25] upon expression in mammalian cells, is a gain of function, resulting in mislocalisation, caused by the frameshifted protein.[26] Overall, these observations do not point to any unifying mechanisms that may explain how specific connexin mutations could cause dominant versus recessive phenotypes. As has been suggested, interactions between connexin isoforms and the effects of connexins on other lens proteins may determine the phenotype.[16 27]

In the present study, the homozygous *GJA8* insertion mutation was associated with a severe phenotype in both affected siblings as indicated by the presence of opacities evident at birth, as well as nystagmus and amblyopia due to severe visual deprivation. One affected family member also had microcornea and microphthalmia, whereas her affected sibling was normal with respect to these parameters. Although it is not possible to conclude here as to whether the occurrence of microphthalmia is causally linked to deficiency of *GJA8*/Cx50, it is worth noting that microphthalmia was a feature of *GJA8* knockout mice, suggesting that it is required for proper growth and development of the eye.[16] This study adds to the range of phenotypes associated with *GJA8* mutations and to our knowledge, describes the first mutation in this gene to be associated with autosomal recessive inheritance of cataract.

Table 2 Cx50 (*GJA8*) mutations reported in human cataracts

Mutation	Phenotype	Reference
Arg23Thr	Progressive congenital nuclear	28
Val44Glu	Congenital or developmental cataract with microcornea	29
Glu48Lys	Zonular nuclear pulverulent	30
Pro88Ser	Zonular pulverulent	19
Pro88Gln	Lamellar pulverulent	21
Val79Leu	"Full moon" with Y-sutural opacity	31
Arg198Glu	Congenital or developmental cataract with microcornea	29
Ile247Met	Zonular pulverulent	32

KEY POINTS

- Candidate gene analysis on an Indian family with autosomal recessive cataract showed an insertion (c.670insA) in *GJA8* that segregated with disease in the family and was consistent with recessive inheritance.
- The mutation is predicted to lead to a frameshift at codon 203 of *GJA8*/connexin 50 with termination after 46 amino acids, giving rise to a protein of 248 residues.
- This study is the first to demonstrate the involvement of connexin 50 in recessive cataract.

ELECTRONIC DATABASE INFORMATION

- Ensembl database: http://www.ensembl.org

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